ARSENIC AND FLUVIAL BIOFILM: BIOGEOCHEMISTRY, TOXICITY AND BIOTIC INTERACTIONS

Laura Barral Fraga

DOCTORAL THESIS

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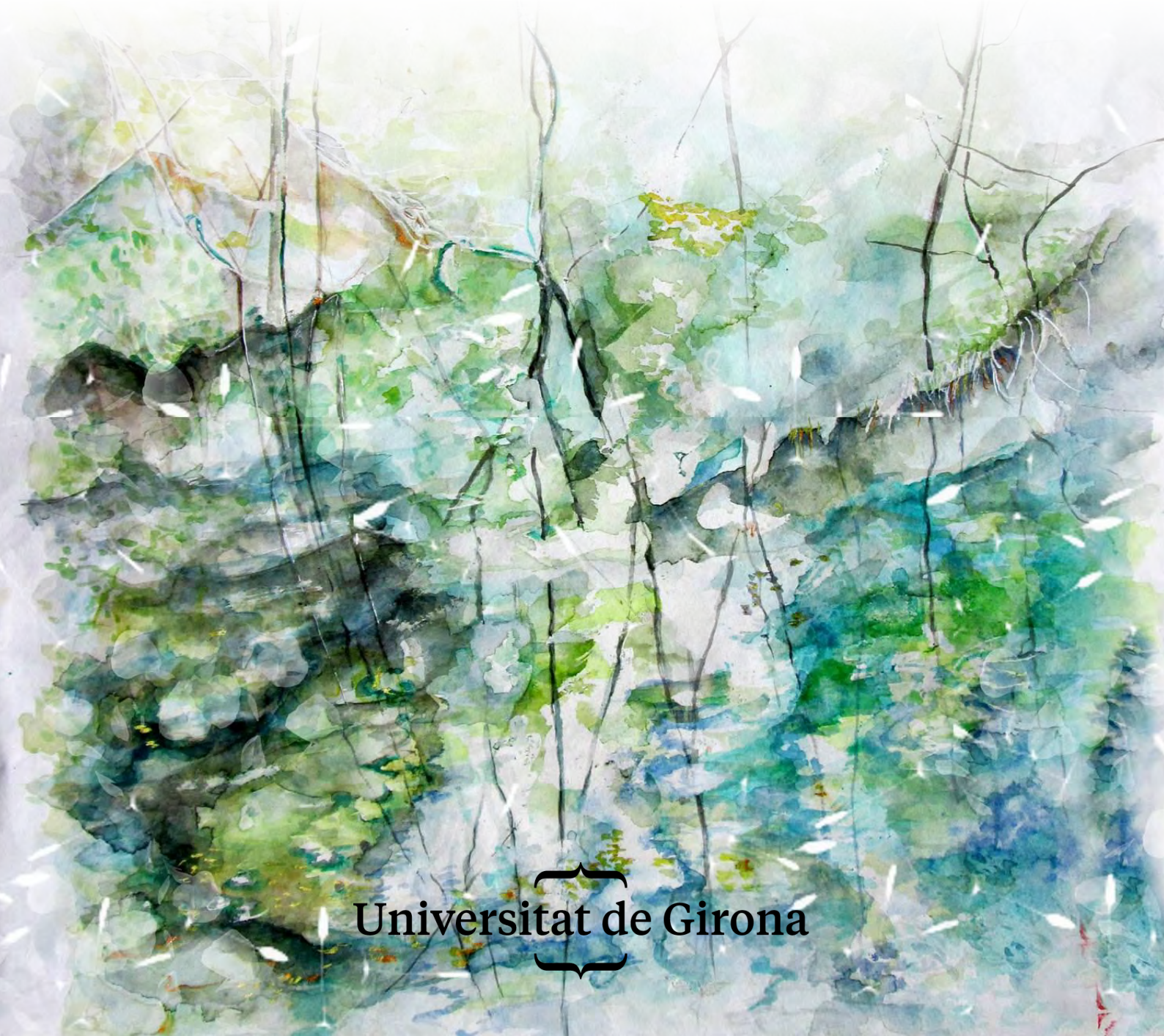

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2017

DOCTORAL PROGRAM IN WATER SCIENCE AND TECHNOLOGY

Supervised by:

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Thesis submitted in fulfillment of the requirements for the Degree of Doctor at the University of Girona

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WE DECLARE:

That the thesis entitled "**Arsenic and Fluvial Biofilms: Biogeochemistry, Toxicity and Biotic Interactions**", presented by **LAURA BARRAL FRAGA** to obtain a Doctoral degree, has been completed under our supervision and meets the requirements to opt for an International Doctorate.

For all intents and purposes, we hereby sign this document.

Signature

Dr. **HELENA GUASCH**	Dr. **MARÍA TERESA BARRAL SILVA**	Dr. **SOIZIC MORIN**

Girona, June 2017

A miña nai

A meu pai

E ós meus avós Manuel e Carmen

Pasa, río, pasa, río,

co teu maino rebulir;

pasa, pasa antre as froliñas

color de ouro e de marfil,

a quen cos teus doces labios

tan doces cousas lle dis.

Pasa, pasa, mais non vexan

que te vas ao mar sin fin,

porque estonces, ¡ai, pobriñas,

cánto choraran por ti!

Rosalía de Castro. *Cantares Gallegos*

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Esta tesis tiene sus inicios allá por el verano 2012 cuando, tras haber defendido el trabajo de fin de master, Helena Guasch me ofrece la posibilidad de seguir trabajando en el campo de la Ecotoxicología comenzando una tesis bajo su supervisión. Y así fue como en pleno 2013 me lancé a la *aventura* que supone ser becaria o investigadora en formación... ☺. Sin embargo, en realidad, esta historia se inició unos cuantos años antes, en los últimos de mi licenciatura en Biología en la Universidade da Coruña, cuando un par de becas de colaboración en departamentos me dieron la oportunidad de descubrir y adentrarme por primera vez en el mundo de la investigación bajo la supervisión de mi gran profesor Roberto Bao. En concreto, fue en ese momento que nació mi fascinación por las diatomeas y la Paleoecología que, junto a mi gran colega David Vázquez, disfruté muchísimo! Inolvidable mi primer muestreo en campo con barca hinchable en la Lagoa costeira de Esteiro con David y Manel Leira!! Gracias Roberto, David y Manel!! Vosotros, junto a Álex Blanco, formaréis siempre parte de los gratos recuerdos que tengo sobre mis inicios en investigación.

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LIST OF PUBLICATIONS

List of publication derived from this Doctoral thesis (two of them are published in international journals):

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doi: http://dx.doi.org/10.1007/s11356-015-4894-8 (see Annex 1)

Magellan K, **Barral-Fraga L**, Rovira M, Srean P, Urrea G, García-Berthou E, Guasch H. (2014). Behavioural and physical effects of arsenic exposure in fish are aggravated by aquatic algae. *Aquatic Toxicology,* 156:116-124.

doi: http://dx.doi.org/10.1016/j.aquatox.2014.08.006 (see Annex 2)

Barral-Fraga L, Martiñá-Prieto D, Barral MT, Morin S, Guasch H. Mutual interaction between arsenic and biofilm in a mining impacted river. *In prep*.

Barral-Fraga L, Barral MT, Prieto D, Rodríguez-Castro MC, Tuulaikhuu B-A, Morin S, Guasch H. Arsenic biogeochemistry in freshwater ecosystems. *In prep*.

LIST OF ACRONYMS

acr3: As^{III} efflux pump-encoding gene
AENOR: Asociación Española de Normalización y Certificación
ANOVA: Analysis of Variance
APHA: American Public Health Association
AQP: aquaglyceroporins
ARMs: arsenate-resistant microorganisms
Arr: arsenate reductase
arrA: arsenate respiratory reductase-encoding gene
Ars: Arsenic resistance system
ArsB: As^{III} efflux pump
ArsB: As^{III} efflux pump
arsB: As^{III} efflux pump-encoding gene
ArsC: arsenate reductase enzyme
arsC: arsenate reductase enzyme-encoding gene
ArsH: arsenate reductase enzyme
arsH: arsenate reductase enzyme-encoding gene
arsM: arsenite methyltransferase enzyme
ArsM: arsenite methyltransferases
arsR: ars operon gen
Arx: anaerobic arsenite oxidation enzyme
As: Arsenic
As-Bet: arsenobetaine
As^{III}: Arsenite
As^{V}: Arsenate
ATP: adenosine triphosphate
CAOs: chemolithoautotrophic arsenite oxidizers
CCC: Criteria Continuous Concentration
Ch: Chapter
Chl-*a*: chlorophyll *a*
CMC: Criteria Maximum Concentration
CTB: chemical time bombs
Cys: cysteine residues
DARPs: Dissimilatory Arsenate-Reducing or Arsenate-Respiring Prokaryotes
df: degrees of freedom
DGT: diffusive gradients in thin films
$DMAA^{III}$: dimethylarsinous acid
$DMAA^{V}$: dimethylarsinic acid
DMA^{III}: dimethylarsenite
DMA^{V}: dimethylarsenate
DNA: Deoxyribonucleic acid
DOC: Dissolved Organic Carbon
DW: Dry Weight
ECOTOX: ECOTOXicology knowledgebase
Eh: Redox potential
EPS: extracellular polymeric substances
F_0: Minimum fluorescence yield
FeAsS: arsenopyrite
Fig.: Figure
GEE: generalized estimating equation
GF/F: glass microfiber filters
GLM: Generalized Linear Model
GSH: glutathione
H: Shannon-Wiener index of diversity
HAOs: heterotrophic arsenite oxidizers
HPLC: High-Performance Liquid Chromatography
iAs: inorganic As
IC20: 20% inhibitory concentration
ICP-MS: Inductively Coupled Plasma Spectrometry
iP: inorganic phosphate
J: Species evenness
L/D: Live/Dead (bacterial viability)
LC50: 50% lethal concentrationLOEL or LOEC: the Lowest Observed Effect Level or the Lowest Observed Effect Concentration
MCL: Maximum Concentration Limit
Met-As: methylarsenicals

μS: microSiemens
$MMAA^{III}$: monomethylarsonous acid
$MMAA^{V}$: monomethylarsonic acid
MMA^{III}: monomethylarsenite
MMA^{V}: monomethylarsenate
MRPP: Multi-Response Permutation Procedures
mV: milliVolts
n: Sample size
NE: North-East
NMDS: Non-Metric Multidimensional Scaling
NOEL or NOEC: No Observable Effect Level or No Observable Effect Concentration
NPL: National Priority List
NW: North-West
OM: organic matter
orgAs: organoarsenicals
P: Phosphorus
p: p-value
PAM: Pulse Amplitude Modulated (fluorimeter)
PAN: Pesticide Action Network
PBET: physiologically based extraction test
PCA: Principal Component Analysis
PCs: phytochelatins
P_i: Proportional abundance of the i^{th} species
p*Ka*: Acid dissociation constant
ppb: parts per billion
PSII: Photosystem II
RDA: Redundancy Rata Analysis
ROS: Reactive Oxygen Species
S: Species richness
SCI: stream and catchment interface
sp.: Species
SPSS: Statistical Package for Social Science (software)
SRP: Soluble reactive phosphorus
SS: Suspended Solids
TC: Total Carbon
TCLP: toxicity characteristic leaching procedure
TMA: trimethylearsine
TMAO: trimethylarsine oxide
TN: Total nitrogen, and Total Kjeldhal nitrogen
TP: Total phosphorus, and Total Phosphate
US EPA: United States Environmental Protection Agency
WHO: World Health Organization
XRF: X-ray fluorescence
Y_{eff}: effective PSII quantum yield or photosynthetic efficiency
Y_{max}: maximum PSII quantum yield or maximal photosynthetic capacity

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4. GENERAL DISCUSSION

CONTENTS

Arsenic (As) contamination of natural waters is a worldwide problem due to its important impacts for human and ecosystem health. Natural (geological processes, mainly) and anthropogenic activities, including mining, are the sources of arsenic pollution in the environment. High concentrations have been reported for water samples in several parts of the world, becoming an environmental concern because of its harmful effects on organisms. Arsenic toxicity depends on numerous interacting factors which makes effects difficult to estimate. In freshwaters, arsenate (As^V) can be taken up by microorganisms (especially those forming biofilms) due to its similarity with phosphate (PO_4^{3-}) molecules, resulting its toxicity be dependent on environmental phosphate conditions. Microorganisms play a key role on the arsenic biogeochemistry (speciation, distribution and cycling) in aquatic systems, since they incorporate the dominant iAs (inorganic arsenic) form and may convert it to other arsenic forms. These transformation reactions have a big impact on the environmental behavior of arsenic, since the different chemical forms of this element exhibit different mobility and toxicity. Fish are another key constituent of aquatic ecosystems, and their effects due to arsenic exposure could be influenced by their interaction with microorganisms (i.e biofilms).

Based on the current knowledge about biofilms ecotoxicology and arsenic biogeochemistry in freshwater ecosystems, this thesis is aiming to study, under realistic environmental arsenic concentrations, **i)** the role of benthic biofilms on As-bioavailability and As-detoxification in a freshwater system, **ii)** the toxic effects of arsenic on the structure and function of benthic fluvial biofilms, with especial attention to diatom responses, and **iii)** the interaction between these As-exposed primary producers and As-exposed higher organisms (fish).

In ***Chapter 1***, an experiment combining ecological and ecotoxicological descriptors was conducted to investigate the effects of As^V (130 µg L^{-1} over 13 days) on the structure and function of fluvial biofilm under phosphate-limiting conditions. We further incorporated fish (*Gambusia holbrooki*) into our experimental system, expecting fish to provide more available phosphate for algae and, consequently, protecting algae against arsenic toxicity. However, this protective role was not fully achieved. Arsenic inhibited algal growth and productivity but not that of bacteria. The diatom community was clearly affected, showing a strong reduction in cell biovolume; selection for tolerant species, in particular *Achnanthidium minutissimum*, and a reduction in species richness. Our results have important implications for risk assessment, as the experimental arsenic concentration used was lower than the acute toxicity criteria established by the United States Environmental Protection Agency (US EPA), 340 µg As L^{-1}.

In ***Chapter 2***, we examined the effects of arsenic exposure (130 µg L^{-1} over 9 days) in the invasive mosquitofish *G. holbrooki*, in the same laboratory experiment as ***Chapter 1***, incorporating some of the complexity of natural systems by including the interacting effects with the microbial community (the biofilm). Our aims were to quantify the effects of arsenic on some complex behaviors and physical parameters in mosquitofish, and to assess whether the detoxifying mechanisms of algae would ameliorate any effects of arsenic exposure. Aggression increased significantly with arsenic whereas neither food capture efficiency nor consumption

was notably affected. Bioaccumulation increased with arsenic and unexpectedly so did fish biomass. Possibly increased aggression facilitated food resource defense allowing bigger fish to gain weight. The presence of algae aggravated the effects of arsenic exposure. For increase in fish biomass, algae acted antagonistically with arsenic, resulting in a disadvantageous reduction in weight gained. For bioaccumulation, the effects were even more severe, as algae operated additively with arsenic to increase arsenic uptake and/or assimilation. Aggression was also highest in the presence of both algae and arsenic. We highlight that multidisciplinary, cross-taxon research, particularly integrating behavioral and other effects, is crucial for understanding the impacts of arsenic toxicity and thus restoration of aquatic ecosystems.

In ***Chapter 3***, a biofilm translocation experiment was carried out during 51 days in a mining-impacted river, the Anllóns River (Galicia, NW Spain), where concentrations up to 270 mg As^{V} kg^{-1} are found in sediments. The translocation was performed moving biofilm-colonized substrata from upstream (less As-polluted) to downstream the mine area (more As-polluted site with also more easily extractable As), to explore the effect of arsenic on benthic biofilms and the role of these biofilms on arsenic retention and speciation in the water-sediment interface. Eutrophic conditions (high total dissolved phosphorus and total nitrogen) were detected in water at both sites, while sediments were not considered P-polluted. Translocated biofilms accumulated more arsenic and showed higher potential toxicity (higher As/P ratio). In concordance, their growth was reduced to half that observed in those non-translocated. Moreover, they became less nutritive (less N content) and with higher bacteria and dead diatom densities than the non-translocated biofilms. Besides the higher arsenic exposure, other environmental conditions such as the higher amount of DOC (dissolved organic carbon) and riparian cover in the more As-polluted site could contribute to those effects. Methylated As-species (DMA^{V}) were found in the intracellular biofilm compartment and also in the river water, suggesting a detoxification process by biofilm (methylation) and a contribution to arsenic speciation in the water-benthic biofilm interface. Since most arsenic in sediments and water was arsenate (As^{V}), the high amount of arsenite (As^{III}) detected in the biofilm extracellular compartment may also confirm As^{V} reduction by biofilms. This study provides new arguments to understand microorganism contribution to arsenic biogeochemistry in freshwater environments.

The results obtained in this thesis provide valuable information to understand the contribution of benthic biofilms to the arsenic biogeochemistry in freshwater environments, and specifically in the water-biofilm interface. Also, it was demonstrated once again the importance of using biofilms and a multi-endpoint approach to measure effects of toxicants in freshwater ecosystems, as well as study the toxicity to different trophic organisms, such as biofilm and fish, since aggravated effects resulted in their interaction. Finally, environmental factors such as nutrients or light may influence and modulate arsenic toxicity. Therefore it is crucial to take them into account for the measurement of real toxic effects in the ecosystems.

La contamination par l'arsenic (As) des eaux naturelles est un problème mondial, avec des impacts importants pour la santé humaine et environnementale. Les activités naturelles (processus géologiques, principalement) et anthropiques, notamment minières, sont les sources principales de pollution à l'arsenic dans l'environnement. Des concentrations élevées ont été rapportées pour des échantillons d'eau collectés dans diverses régions du monde, ces niveaux étant préoccupants en raison d'effets néfastes sur les organismes. La toxicité de l'arsenic dépend de nombreux facteurs en interaction, ce qui rend difficile la prédiction des effets. Dans les eaux douces, l'arséniate (As^V) peut être accumulé par des micro-organismes (notamment les biofilms) en raison de sa similitude avec la molécule de phosphate (PO_4^{3-}); sa toxicité dépend donc des concentrations environnementales en phosphate. Les micro-organismes jouent un rôle clé sur la biogéochimie de l'arsenic (spéciation, distribution et recyclage) dans les systèmes aquatiques, car ils peuvent convertir la forme dominante iAs (arsenic inorganique) en d'autres formes de l'arsenic. Ces réactions de transformation ont un impact important sur le comportement environnemental de l'arsenic, car les différentes formes chimiques de cet élément présentent une mobilité et une toxicité différentes. Les poissons sont une autre composante clé des écosystèmes aquatiques, et les effets causés par l'exposition d'arsenic pourraient être influencés par la présence et l'action des micro-organismes (i.e. les biofilms).

Sur la base des connaissances actuelles sur l'écotoxicologie des biofilms et de la biogéochimie de l'arsenic dans les écosystèmes d'eau douce, cette thèse vise à étudier, en présence de concentrations environnementalement réalistes **i)** le rôle des biofilms benthiques sur la biodisponibilité de l'arsenic et la détoxification dans un système d'eau douce, **ii)** les effets toxiques de l'arsenic sur la structure et la fonction des biofilms benthiques de rivière, avec une attention particulière portée aux réponses des diatomées, et **iii)** l'interaction entre ces producteurs primaires exposés à l'arsenic et des organismes supérieurs (poissons) également exposés à l'arsenic.

Dans le ***Chapitre 1***, une expérimentation combinant des descripteurs écologiques et écotoxicologiques a été réalisée pour étudier les effets de l'arsenic (130 µg L^{-1} pendant 13 jours) sur la structure et la fonction des *biofilms* fluviaux dans des conditions limitantes en phosphate. Nous avons intégré en plus des poissons (*Gambusia holbrooki*) dans notre système expérimental, en faisant l'hypothèse que la présence des poissons fourniraient plus de phosphate disponible pour les algues et, par conséquent, protégeraient les algues contre la toxicité de l'arsenic. Cependant, ce rôle protecteur n'a pas été pleinement atteint. L'arsenic a inhibé la croissance et la productivité des algues, mais pas celle des bactéries. La communauté de diatomées a été affectée, montrant une forte réduction du biovolume cellulaire, une sélection des espèces tolérantes, en particulier *Achnanthidium minutissimum*, et une réduction de la richesse spécifique. Nos résultats ont des implications importantes pour l'évaluation des risques liés à l'arsenic, car la concentration expérimentale d'arsenic utilisée était plus faible que les critères de toxicité aigue établis par l'US EPA, à savoir 340 µg As L^{-1}.

Dans le ***Chapitre 2***, nous avons examiné les effets de l'exposition à l'arsenic (130 µg L^{-1} pendant 9 jours) sur la gambusie invasive *G. holbrooki*, dans la même expérimentation de laboratoire que le ***Chapitre 1***, intégrant une partie de la complexité des systèmes naturels au travers de l'interaction avec la communauté microbienne (le biofilm). Nos objectifs étaient de quantifier les effets de l'arsenic sur certains comportements complexes de la gambusie et sur ses paramètres physiques, et d'évaluer si les mécanismes de détoxification de l'arsenic par les algues réduisaient les effets de l'exposition à l'arsenic. En présence d'arsenic, le comportement agressif des poissons a augmenté significativement, tandis que ni l'efficacité de capture de nourriture, ni la consommation, n'ont été affectées par la présence d'arsenic. Une augmentation de la bioaccumulation a été observée avec l'exposition à l'arsenic ainsi que, de façon inattendue, de la biomasse de poissons. Il est possible que la stimulation du comportement d'agression ait par ailleurs facilité l'accès aux ressources alimentaires, permettant aux plus gros poissons de prendre du poids. Une aggravation des effets de l'exposition à l'arsenic a été démontrée en présence d'algues. Concernant la biomasse de poissons, la présence d'algues a agi de manière antagoniste avec l'arsenic, entraînant une réduction du poids final. Concernant la bioaccumulation, les effets ont été encore plus marqués, avec des effets additifs de la présence d'algues et de l'arsenic sur l'augmentation de l'absorption et/ou de l'assimilation d'arsenic dans les poissons. Enfin, les comportements d'agression étaient la plus élevés en présence des algues et de l'arsenic. Nous mettons en évidence qu'une recherche multidisciplinaire, utilisant des organismes de différents niveaux trophiques, et considérant les effets comportementaux en plus d'autres effets plus classiquement évalués, est essentielle pour comprendre les impacts de la toxicité de l'arsenic, et donc pour contribuer à la restauration des écosystèmes aquatiques.

Dans le ***Chapitre 3***, une expérimentation de translocation de biofilm a été réalisée pendant 51 jours, dans une rivière impactée par l'exploitation minière: la rivière Anllóns (Galice, nord-ouest de l'Espagne) où les sédiments présentent des concentrations allant jusqu'à 270 mg As^{V} kg^{-1}. Des substrats précolonisés par du biofilm au site amont (moins pollué par l'arsenic) ont été ensuite maintenus sur place ou déplacés dans une zone en aval de la mine (le site le plus pollué par l'arsenic, avec notamment dans le sédiment des formes facilement extractibles de l'arsenic), pour explorer l'effet de l'arsenic sur les biofilms benthiques, et le rôle de ces *biofilms* sur la rétention et la spéciation de l'arsenic à l'interface eau-sédiment. Dans les deux sites, les eaux présentaient des conditions eutrophes (concentrations élevées en phosphore dissous et en azote total), alors que les sédiments n'étaient pas considérés comme pollués au regard de leur teneur en phosphore. Les biofilms transloqués ont accumulé plus d'arsenic et les rapports As/P mesurés, plus élevés, y suggèrent une toxicité potentielle accrue. En concordance, leur croissance a été réduite de moitié, en comparaison avec les biofilms amont. De plus, ils sont devenus moins nutritifs (avec une teneur plus faible en N), avec une augmentation de la densité de bactéries et de diatomées mortes par rapport aux les biofilms non déplacés. En plus des conditions d'exposition à des concentrations élevées en arsenic,

d'autres conditions environnementales, telles que la teneur plus élevée en carbone organique dissous et la couverture végétale riveraine du site, pourraient contribuer à ces effets. L'espèce méthylée de l'arsenic DMA^V a été trouvée dans le compartiment intracellulaire du biofilm ainsi que dans l'eau de la rivière, ce qui suggère un processus de détoxification de l'arsenic par les biofilms (méthylation), et une contribution à la spéciation d'arsenic à l'interface eau-biofilm benthique. L'espèce dominante dans les sédiments et dans l'eau étant l'As^V, les quantités élevées d'arsénite (As^{III}) détectées dans le compartiment extracellulaire du biofilm peuvent également confirmer la réduction en As^V par les biofilms.

Les résultats obtenus dans cette thèse fournissent des informations précieuses pour comprendre la contribution des biofilms benthiques à la biogéochimie de l'arsenic dans les milieux d'eau douce, et plus précisément à l'interface eau-biofilm. En outre, ces travaux confirment l'importance de l'utilisation de biofilms et d'une approche multi-descripteurs pour évaluer les effets des composés toxiques dans les écosystèmes d'eau douce. L'intérêt de considérer dans les études écotoxicologiques les interactions entre différents organismes de l'édifice trophique, tels que les biofilms et les poissons, a été également démontré, car la présence conjointe des algues et de l'arsenic dans les systèmes expérimentaux s'est accompagnée d'une aggravation des effets observés sur le maillon trophique supérieur. Enfin, les facteurs environnementaux tels que la lumière ou les nutriments peuvent influencer et moduler la toxicité, il est donc crucial de les prendre en compte pour une meilleure évaluation des effets réels des toxiques sur les écosystèmes.

A contaminación por arsénico (As) nas augas naturais é un problema global por mor dos seus impactos significativos na saúde humana e nos ecosistemas. Os procesos naturais (procesos xeolóxicos, principalmente) e antropoxénicos, como a minería, son fontes de contaminación por arsénico no medio ambiente. Téñense atopado elevadas concentracións deste metaloide en mostras de auga de varias partes do mundo, tornándose unha preocupación ambiental por mor dos seus efectos nocivos sobre os organismos. A toxicidade do arsénico depende de moitos factores que interactúan entre sí, o que fai que os seus efectos sexan difíciles de estimar. Nas augas doces, o arsenato (As^V) pode ser absorbido por microorganismos (*biofilm*s ou biofilmes), debido á súa semellanza coa molécula dun nutrinte, o fosfato (PO_4^{3-}), dependendo así a súa toxicidade das concentracións ambientais de fosfato. Os microorganismos xogan un papel fundamental na bioxeoquímica do arsénico bioquímico (é dicir, na súa especiación, distribución e no seu ciclo) en sistemas acuáticos, xa que incorporan a forma dominante de iAs (arsénico inorgánico), que soe ser o arsenato, podendo despois convertelo noutras formas de arsénico. Estas reaccións de transformación teñen un impacto importante sobre o comportamento ambiental do arsénico, porque diferentes formas químicas deste metaloide teñen tamén diferente mobilidade e toxicidade. Os peixes son outro compoñente clave dos ecosistemas acuáticos, e os efectos debidos á súa exposición ao arsénico poderían verse influenciados ao interactuaren cos *biofilms*).

Baseándose no coñecemento actual sobre a ecotoxicoloxía dos *biofilms* e a bioxeoquímica do arsénico nos ecosistemas de augas doces, esta tese pretende estudar, empregando concentracións ambientais realistas, **i)** o papel dos *biofilm*s bentónicos na biodispoñibilidade e desintoxicación do arsénico nun sistema de auga doce, **ii)** os efectos tóxicos do arsénico sobre a estrutura e a función dos *biofilm*s fluviais, con especial atención ás diatomeas (microalgas marróns), e **iii)** a interacción entre estes produtores primarios e organismos superiores coma os peixes cando se ven todos eles expostos a este metaloide.

Así pois, no ***capítulo 1*** levouse a cabo un experimento combinando descritores ecolóxicos e ecotoxicolóxicos, para investigar os efectos do As^V (130 µg L^{-1} durante 13 días) sobre a estrutura e a función do *biofilm* fluvial en condicións de limitación de fosfato. Ademáis, incorporáronse peixes (o mosquitofish oriental *Gambusia holbrooki*) no sistema experimental, esperando que puidesen proporcionar máis fosfato ás algas a través das súas excrecións e, polo tanto, protexelas contra a toxicidade do arsénico. Con todo, este papel protector non foi alcanzado por completo, pois o arsénico inhibiu o crecemento e a produtividade algal, anque non o crecemento das bacterias. A comunidade de diatomeas viuse claramente afectada, mostrando unha forte redución no seu biovolume celular e unha especial selección cara especies tolerantes -particularmente *Achnanthidium minutissimum*- causando, polo tanto, unha redución no número de especies (menor riqueza específica). Os nosos resultados teñen implicacións importantes para a avaliación dos riscos ambientais do arsénico, xa que a concentración utilizada neste experimento (130 µg L^{-1}) foi inferior aos criterios de toxicidade

aguda establecidos pola Axencia de Protección Ambiental dos Estados Unidos (US EPA), 340 µg As L^{-1}.

No ***capítulo 2***, preséntanse os resultados dun experimento de laboratorio (o mesmo que no ***capítulo 1***) no que se examinaron os efectos do arsénico (130 µg L^{-1} durante 9 días) sobre o peixe *G. holbrooki*, unha especie invasora. O experimento incorporou parte da complexidade dos sistemas naturais incluíndo a interacción dos peixes coa comunidade microbiana (*biofilm*). O noso obxectivo foi cuantificar os efectos do arsénico no peixe analizando algúns comportamentos complexos e parámetros físicos, e avaliar o papel detoxificador do biofilm. A agresividade dos peixes aumentou significativamente en presenza de arsénico, mentres que nin a eficiencia de capturas dos alimentos nin o consumo dos mesmos se viron afectados polo dito tóxico. Observouse unha maior acumulación de arsénico nos peixes e, de forma inesperada, un aumento do seu peso (biomasa) no tratamento con arsénico. Probablemente, o aumento da agresividade facilitou o acceso aos recursos alimenticios, permitindo que os peixes máis grandes gañasen máis peso. O máis salientable é que a presenza de *biofilm* agravou os efectos da exposición ao arsénico en peixes. En canto á biomasa dos peixes, o *biofilm* actuou de forma antagónica co arsénico, resultando na redución desvantaxosa de peso nos peixes. En canto á bioacumulación, os efectos foron aínda máis graves, xa que na presenza de *biofilm* a captación e/ou asimilación do arsénico nos peixes aumentou. A agresividade nestes animais resultou tamén ser máis forte na presenza de arsénico e *biofilm*. Queremos salientar a importancia da investigación de tipo multidisciplinaria, na que se teña en conta a interacción entre distintos organismos da rede trófica, e integrando o estudo de diferentes efectos sobre os organismos (coma os cambios no comportamento, por exemplo, entre outros), sendo crucial para entender mellor os impactos reais do arsénico nos ecosistemas acuáticos.

O ***capítulo 3*** baséase nun experimento de translocación de *biofilm* levado a cabo durante 51 días no río Anllóns (Galicia), o cal se acha afectado pola actividade mineira, podéndose atopar ata 270 mg kg^{-1} de As^{V} nos seus sedimentos. A translocación realizouse movendo substratos colonizados con *biofilm* dende un tramo do río situado augas arriba da zona mineira (menos contaminada) a un tramo augas abaixo da mesma (máis contaminado e cunha maior proporción da fracción máis móbil de arsénico). O experimento tiña un doble obxetivo: i) examinar o efecto do arsénico sobre o *biofilm* bentónico, e ii) o papel deste *biofilm* na retención e especiación do arsénico na interface auga-sedimento. Detectáronse condicións eutróficas na auga de ámbolos dous tramos (concentracións elevadas de fósforo total disolto e de nitróxeno total), mentres que os sedimentos non se atoparon contaminados por fósforo. Os *biofilms* do tramo máis contaminado acumularon máis arsénico e mostraron unha maior toxicidade potencial (maior relación As/P). Por conseguinte, o seu crecemento viuse reducido á metade do observado nos *biofilms* do tramo menos contaminado.Ditos *biofilms* perderon calidade nutricional (menor contido de N) e mostraron unha maior densidade de bacterias e diatomeas mortas ca nos *biofilm*s non translocados. Ademáis da exposición ao arsénico, outras

condicións ambientais coma o carbono orgánico disolto ou a cuberta do bosque de ribeira (superiores no tramo situado augas abaixo) poderían explicar os efectos observados. En canto ao efecto do *biofilm* sobre o arsénico, a presenza de especies químicas metiladas e menos tóxicas (DMA^V) tanto na auga coma no interior das células do *biofilm*, indican que o biofilm contribuiu á especiación do arsénico na interface auga-*biofilm* bentónico, reducindo a súa toxicidade. Por outra banda, xa que a maior parte do arsénico en sedimentos e auga é arsenato (As^V), a gran cantidade de arsenito (As^{III}) detectado no compartimento extracelular confirmaría a redución de As^V por parte deste *biofilm*.

Os resultados obtidos nesta tese proporcionan información valiosa para comprender a contribución dos *biofilm*s á bioxeoquímica do arsénico en ambientes de auga doce e, especialmente, na interface auga-*biofilm*. Unha vez máis, vólvese a demostrar a importancia do uso dos *biofilm*s e cun enfoque multi-resposta para avalia-la magnitude dos efectos dos contaminantes (substancias tóxicas) sobre os ecosistemas de auga doce. Queremos salientar tamén o valor dos estudos de toxicidade nas interaccións entre diferentes organismos tróficos, coma os *biofilm*s o os peixes, xa que os efectos máis graves observados nestes organismos superiores resultaron desta interacción. Finalmente, os estudos de campo mostran que a resposta dos organismos aos factores ambientais (coma a luz ou a concentración de nutrintes) pode enmascarar o efecto dos contaminantes, polo que é fundamental tomalos en consideración.

La contaminació per arsènic (As) en el medi aquàtic és considerada un problema a nivell mundial, degut als seus efectes sobre la salut humana i la dels ecosistemes. Aquesta contaminació prové de processos naturals (principalment geològics) i d'activitats antropogèniques, com la mineria. En diverses parts del món, se n'han trobat concentracions elevades, esdevenint un problema ambiental. Si més no, la toxicitat de l'arsènic és difícil d'estimar ja que depèn de la interacció entre nombrosos factors. En aigües dolces, l'arseniat (As^V) pot ser absorbit pels microorganismes (especialment pels biofilms) a causa de la seva similitud amb el fosfat (PO_4^{3-}), sent la seva toxicitat depenent de la concentració de fosfat. Els microorganismes tenen un paper clau en la biogeoquímica de l'arsènic (especiació, distribució i cicle) en els sistemes aquàtics, ja que n'incorporen la forma dominant, que és el iAs (arsènic inorgànic) i poden convertir-lo en altres formes químiques. Aquestes reaccions de transformació tenen un gran impacte en el seu comportament ambiental, ja que les diferents formes químiques d'aquest element difereixen en quan a la seva mobilitat i toxicitat. Els peixos són un altre element clau dels ecosistemes aquàtics, sensibles a la presència d'arsènic, la toxicitat del qual pot estar influenciada per la seva interacció amb els microorganismes (és a dir, els biofilms).

Basant-nos en els coneixements actuals sobre ecotoxicologia dels biofilms i biogeoquímica de l'arsènic en ecosistemes d'aigua dolça, aquesta tesi té com a objectius estudiar, en concentracions ambientals i realistes d'arsènic, ***i)*** el paper dels biofilms bentònics en la biodisponibilitat i de-toxicació de l'arsènic en un sistema d'aigua dolça, ***ii)*** els efectes tòxics de l'arsènic en l'estructura i funció dels biofilms fluvials bentònics, fent especial atenció a la resposta de les diatomees, i ***iii)*** la interacció entre els productors primaris i altres organismes superiors (peixos) quan es troben sota l'efecte de l'arsènic.

Al ***capítol 1***, s'exposen els resultats d'un experiment en el que es van investigar els efectes del As^V (130 µg L^{-1} durant 13 dies) en l'estructura i funció del biofilm fluvial en condicions de limitació de fosfat. A més, es van incorporar peixos (*Gambusia holbrooki*) a l'experiment, esperant que aquests proporcionessin més fosfat a les algues i, en conseqüència, les protegissin de la toxicitat de l'arsènic. No obstant això, no es va aconseguir plenament aquesta funció protectora. L'arsènic va inhibir el creixement algal i la seva productivitat. Per altra banda, els bacteris no es van veure afectats. L'arsènic va afectar de manera clara a la comunitat de diatomees, mostrant una forta reducció del biovolum cel·lular; una selecció d'espècies tolerants, en particular *Achnanthidium minutissimum*, i una reducció en la riquesa d'espècies. Els nostres resultats tenen implicacions importants per a l'avaluació dels riscos ambientals de l'arsènic, ja que la concentració utilitzada en aquest experiment (130 µg As L^{-1}) és inferior als criteris de toxicitat aguda establerts per l'Agència de Protecció Ambiental dels Estats Units (US EPA), 340 µg As L^{-1}.

Al ***capítol 2***, es presenten els resultats d'un experiment de laboratori (el mateix que al ***capítol 1***) en el que es van examinar els efectes de l'arsènic (130 µg L^{-1} durant 9 dies) sobre el peix *G. holbrooki,* una espècie invasora. L'experiment incorpora part de la complexitat dels

sistemes naturals mitjançant la inclusió dels efectes interactius del tòxic amb la comunitat microbiana (el biofilm). El nostre objectiu va ser quantificar els efectes de l'arsènic en alguns comportaments complexos i paràmetres físics dels peixos, i avaluar el paper de-toxificador del biofilm. L'agressivitat dels individus de gambúsia va augmentar de manera significativament amb l'arsènic mentre que el moviment de l'opercle disminuí lleugerament (de manera no significativa). A més, ni l'eficiència de captura dels aliments ni el consum es van veure afectats pel tractament amb arsènic. L'arsènic es va bioacumular de manera significativa. Per altra banda, el pes (biomassa) dels peixos va augmentar en el tractament amb arsènic, resultat que no havíem anticipat. Possiblement, l'augment de l'agressió va facilitar l'accés a l'aliment, fent que els peixos guanyessin més pes. La presència de biofilm va alterar la resposta dels peixos a l'arsènic, actuant antagònicament. Pel que fa a la bioacumulació, els efectes van ser encara més greus, ja que en presència de biofilm la captació i/o assimilació d'arsènic va incrementar. L'agressivitat en aquests animals va ser també més important en la presència de biofilm i arsènic. Volem destacar la importància d'una investigació de tipus multidisciplinària, en la qual es tingui en compte la interacció entre diferents organismes de la xarxa tròfica, i integrant l'estudi de diferents efectes sobre els organismes (com els canvis en el comportament, per exemple, entre d'altres), sent crucial per entendre millor els impactes reals de l'arsènic en els ecosistemes aquàtics.

El ***capítol 3*** es basa en un experiment de translocació de biofilm dut a terme durant 51 dies en un riu gallec afectat de l'activitat minera, el riu Anllóns, que conté concentracions de fins a 270 mg As^{V} kg^{-1} en el sediment. La translocació va consistir en transportar substrats colonitzats amb biofilm des d'un tram de riu situat aigües amunt de la zona minera (amb menor contaminació d'arsènic) a un altre tram situat aigües avall (més contaminat i amb una proporció més gran de la fracció més mòbil d'arsènic). L'experiment tenia un doble objectiu *i)* examinar l'efecte de l'arsènic sobre el biofilm bentònic, i *ii)* analitzar el paper d'aquest biofilm sobre la retenció i especiació de l'arsènic en la interfície aigua-sediment. Es van detectar condicions eutròfiques (concentracions elevades de fòsfor dissolt total i nitrogen total a l'aigua) a ambdós trams, si bé els sediments no estaven contaminats amb fòsfor. Els *biofilms* translocats van acumular més arsènic i van mostrar una major toxicitat potencial (major relació de As/P). En concordança, el seu creixement es va reduir a la meitat de l'observat en els biofilms del tram menys contaminat. A més, el biofilm es va fer menys nutritiu (menor contingut de N) i va augmentar el nombre de bacteris i la densitat de diatomees mortes en relació amb el biofilm no translocat. A més de l' exposició a l'arsènic, altres condicions ambientals, com ara el carboni orgànic dissolt (DOC) i la cobertura del bosc de ribera (superiors al tram situat aigües avall) podrien explicar els efectes observats. En relació amb l'efecte del biofilm sobre l'arsènic, la presència d'espècies químiques metilades d'arsènic (DMA^{V}) tant a l'aigua del riu com a l'interior de les cèl·lules del biofilm, indiquen que el biofilm contribueix a l'especiació de l'arsènic en la interfície aigua-biofilm bentònic reduint-ne la seva toxicitat (ja que la forma metilada té menor toxicitat). Per altra banda, atès que la major part de l'arsènic en aigua i en els sediments és

arseniat (As^{V}), l'elevada quantitat de arsenit (As^{III}) detectat en el compartiment extracel·lular del biofilm confirmaria la reducció d'As^{V} per part del biofilm.

Els resultats obtinguts en aquesta tesi proporcionen informació valuosa per comprendre la contribució del biofilm a la biogeoquímica de l'arsènic en ambients d'aigua dolça, i especialment a la interfície biofilm-aigua. A més, es va demostrar una vegada més la importància de l'ús de biofilms i amb un enfoc multi-resposta per avaluar la magnitud dels efectes dels contaminants (substàncies tòxiques) sobre els ecosistemes d'aigua dolça. També volem remarcar en el valor dels estudis de toxicitat en les interaccions entre els diferents organismes tròfics, com ara els biofilms o els peixos, ja que els efectes més greus observats en aquests organismes superiors van ser resultat d'aquesta interacció. Finalment, els estudis de camp ens mostren que la resposta dels organismes als factors ambientals (la llum o la concentració de nutrients) pot emmascarar l'efecte dels contaminants, pel que cal tenir-los en compte.

La contaminación por arsénico (As) de las aguas naturales es un problema mundial debido a sus importantes impactos en la salud humana y en los ecosistemas. Los procesos naturales (procesos geológicos, principalmente) y antropogénicos, como la minería, son las fuentes de contaminación por arsénico en el medio ambiente. Se han publicado altas concentraciones de arsénico en muestras de agua de varias partes del mundo, convirtiéndose en una preocupación ambiental debido a sus efectos nocivos sobre los organismos. La toxicidad del arsénico depende de numerosos factores que interactúan entre sí, lo que hace que los efectos sean difíciles de estimar. En aguas dulces, el arseniato (As^V) puede ser absorbido por microorganismos (especialmente como biofilms) debido a su similitud con la molécula de fosfato (PO_4^{3-}), siendo su toxicidad dependiente de las condiciones ambientales de este nutriente. Los microorganismos desempeñan un papel clave en la biogeoquímica del arsénico (en su especiación, distribución y ciclo) en los sistemas acuáticos, ya que incorporan la forma dominante de iAs (arsénico inorgánico), que suele ser el arseniato, y pueden convertirla en otras formas de arsénico. Estas reacciones de transformación tienen un gran impacto en el comportamiento ambiental del arsénico, ya que las diferentes formas químicas de este elemento presentan diferente movilidad y toxicidad. Los peces son otro componente clave de los ecosistemas acuáticos, y sus efectos debidos a la exposición al arsénico podrían verse influidos por su interacción con los microorganismos (es decir, con biofilms).

Basándonos en los conocimientos actuales sobre la ecotoxicología del biofilm y la biogeoquímica del arsénico en los ecosistemas de agua dulce, esta tesis pretende estudiar, bajo concentraciones ambientales realistas, **i)** el papel de los biofilms bentónicos en la biodisponibilidad y destoxificación del arsénico en un sistema de agua dulce, **ii)** los efectos tóxicos del arsénico sobre la estructura y función de los biofilms bentónicos fluviales, prestando especial atención a las respuestas de las diatomeas, y **iii)** la interacción entre estos productores primarios y organismos superiores como los peces cuando se encuentran bajo el efecto del arsénico.

Así, en el ***capítulo 1*** se realizó un experimento que combinaba descriptores ecológicos y ecotoxicológicos para investigar los efectos del As^V (130 µg L^{-1} durante 13 días) sobre la estructura y función del biofilm fluvial y bajo condiciones de limitación de fosfato. Además incorporamos peces (*Gambusia holbrooki*) en nuestro sistema experimental, esperando que pudiesen proporcionar más fosfato a las algas y, en consecuencia, protegerlas contra la toxicidad de arsénico. Sin embargo, este papel protector no se logró por completo. El arsénico inhibió el crecimiento y la productividad de las algas, pero no el de las bacterias. La comunidad de diatomeas fue claramente afectada mostrando una fuerte reducción en el biovolumen celular y una selección de especies tolerantes -en particular *Achnanthidium minutissimum*- causando, por tanto, una reducción en la riqueza de especies. Nuestros resultados tienen implicaciones importantes para la evaluación de los riesgos ambientales del arsénico, ya que la concentración experimental utilizada de este elemento fue menor que la concentración límite establecida por la Agencia de Protección Ambiental de los Estados Unidos (US EPA) para la toxicidad aguda, 340 µg As L^{-1}.

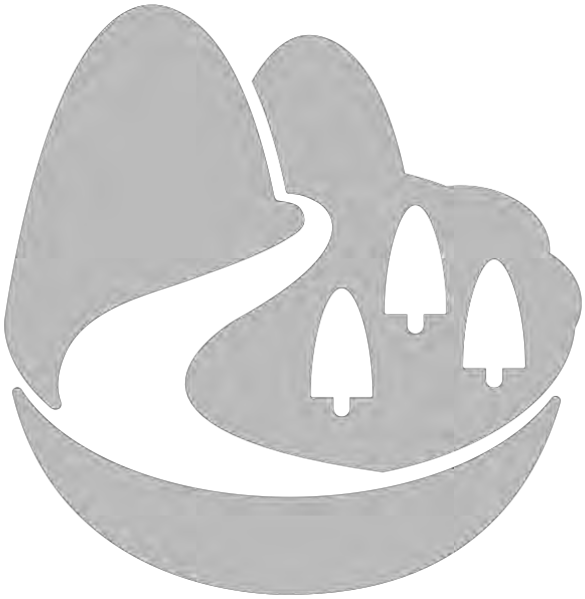
En el ***capítulo 2***, se examinaron los efectos de la exposición al arsénico en el pez mosquito *G. holbrooki* (130 µg L^{-1} a lo largo de 9 días) en un experimento de laboratorio (el mismo que en el ***capítulo 1***) que incorporaba parte de la complejidad de los sistemas naturales al incluir los efectos interactivos del tóxico con la comunidad microbiana (el biofilm). Nuestro objetivo era cuantificar los efectos del arsénico sobre algunos comportamientos complejos y sobre parámetros físicos en los peces y evaluar el papel detoxificador del biofilm. La agresividad aumentó significativamente en presencia de arsénico, mientras que el movimiento opercular disminuyó de forma no significativa, y ni la eficiencia ni el consumo de la captura de alimentos se vieron notablemente afectados. La bioacumulación aumentó con el arsénico y, de forma inesperada, también lo hizo la biomasa de los peces. Posiblemente el aumento de la agresividad facilitó la defensa por los recursos alimentarios permitiendo que los peces más grandes aumentaran de peso. Lo más destacable es que la presencia de biofilm agravó los efectos de la exposición al arsénico en los peces. En cuanto al aumento de la biomasa de peces, el biofilm actuó de forma antagónica con el arsénico, dando como resultado una reducción desventajosa del peso ganado en los peces. En cuanto a la bioacumulación, los efectos fueron aún más graves, ya que las algas también contenían arsénico y, por tanto, proporcionaban un aumento de absorción y/o asimilación de arsénico en los peces. La agresividad en estos animales resultó también más importante en presencia de algas y arsénico. Queremos destacar la importancia de una investigación de tipo multidisciplinaria, en la que se tenga en cuenta la interacción entre distintos organismos de la red trófica, e integrando el estudio de distintos efectos posibles en los organismos (como los cambios en el comportamiento, por ejemplo, entre otros), siendo fundamental para entender mejor los impactos reales del arsénico en los ecosistemas acuáticos.

En el ***capítulo 3***, se realizó un experimento de translocación de biofilm durante 51 días en un río impactado por la minería, el río Anllóns (Galicia, noroeste de España), donde se suelen encontrar concentraciones de hasta 270 mg As^{V} kg^{-1} en sus sedimentos. La translocación se realizó moviendo los sustratos colonizados por biofilm desde aguas arriba (menos contaminado) hasta aguas abajo del área de la mina (en el punto más contaminado por arsénico y, además, más fácilmente extraíble del sedimento al agua), para explorar el efecto del arsénico en biofilms bentónicos y el papel de estos biofilms sobre la retención y especiación de arsénico en la interfaz agua-sedimento. Se detectaron condiciones eutróficas (altos niveles de fósforo total disuelto y nitrógeno total) en el agua en ambos puntos de muestreo, mientras que los sedimentos no se consideraron contaminados por fósforo. Los biofilms translocados acumularon más arsénico y presentaron mayor toxicidad potencial (mayor relación As/P). En concordancia, su crecimiento se redujo a la mitad de lo observado en aquellos no translocados. Además, perdieron calidad nutricional (menor contenido de N) y mostraron mayor densidad de bacterias y de diatomeas muertas que los biofilms no translocados. A mayores de la alta concentración de arsénico a la que estaban expuestos, otras condiciones ambientales tales como una mayor cantidad de carbono orgánico disuelto y de cubierta ribereña en el sitio de muestreo situado aguas abajo de la mina podrían contribuir a dichos efectos. En el

compartimiento intracelular de los biofilms, así como en el agua del río, se encontraron especies de arsénico metiladas menos tóxicas (principalmente, DMA^{V}), indicando que el *biofilm* contribuyó a la especiación del arsénico en la interfaz agua-biofilm bentónico, reduciendo así su toxicidad. Dado que la mayoría de arsénico en sedimentos y agua era As^{V}, la gran cantidad de arsenito (As^{III}) detectada en el compartimiento extracelular de estos biofilms puede también confirmar la existencia de un proceso de reducción de As^{V} por biofilms.

Los resultados obtenidos en esta tesis proporcionan información valiosa para comprender la contribución de los biofilms a la biogeoquímica del arsénico en ambientes de agua dulce y, específicamente, en la interfaz agua-biofilm. Una vez más, se volvió a demostrar la importancia del uso del biofilm y con un enfoque multi-respuesta para evaluar la magnitud de los efectos de los contaminantes (substancias tóxicas) sobre los ecosistemas de agua dulce. También queremos destacar el valor del estudio de la toxicidad en las interacciones entre diferentes organismos tróficos, como los biofilms y los peces, ya que los efectos más graves observados en los peces resultaron de esta interacción. Finalmente, los estudios de campo muestran que la respuesta de los organismos a los factores ambientales (como la luz o la concentración de nutrientes) puede enmascarar el efecto de los contaminantes, por lo que es fundamental tomarlos en consideración.

1. General Introduction



1. ARSENIC OCCURENCE AND FATE IN FRESHWATER ENVIRONMENTS

Arsenic (As) is a major environmental pollutant (Singh *et al.* 2007), widely dispersed in the Earth's crust (Garelick *et al.* 2009), where it ranks as the 20th most abundant trace element (NRC 1977). It is also widely distributed in soil, freshwater and marine environments, with no known biological roles (Wang *et al.* 2015). Arsenic contamination of natural waters (groundwater, seawater and freshwater) is a worldwide problem as high concentrations have been reported for water samples in several parts of the world (Smedley and Kinniburgh 2001; 2002; Rahman *et al.* 2012). Moreover, arsenic contamination of natural waters is one of the major environmental concerns because of its harmful effects on organisms, directly by ingestion and inhalation, or indirectly through the food chain pathways (Rahman and Hasegawa 2012). The Aquatic Life Criteria (United States Environmental Protection Agency, US EPA 2014) establishes limits for arsenic concentration in freshwaters: the Criteria Maximum Concentration (CMC), which refers to acute arsenic exposure, is set at 340 μg As L^{-1}; while the Criteria Continuous Concentration (CCC), which refers to chronic arsenic exposure, is set at 150 μg As L^{-1}. A much lower concentration (10 μg As L^{-1}) was established as the maximum concentration limit (MCL) for drinking water by the World Health Organization (WHO, 1993).

1.1 Arsenic sources

Arsenic is a constituent of more than 200 minerals (Garelick *et al.* 2009) and is primarily present in the form of chemically reduced minerals, like realgar (AsS), orpiment (As_2S_3) and arsenopyrite (FeAsS), the latter being the most abundant arsenic ore (Smedley and Kinniburgh 2002).

The fate of arsenic in freshwater systems is similar to that of some metals and other metalloids (Fig. 1). Natural processes such as volcanic emissions, rock weathering and biological activity are responsible for the occurrence and distribution of arsenic in the environment. Arsenic enters the atmosphere through dust particles coming from volcanic emissions (ashes), wind erosion, low-temperature volatilization from soils, marine aerosols and pollution, and is returned to the Earth’s surface (mainly to water bodies) by atmospheric precipitation; then, it moves through terrestrial runoff and groundwater discharge. Once in water, it binds to organic and inorganic particles, and tends to sink to the sediments (Belzile and Morris 1995; Smedley and Kinniburgh 2002; Rahman and Hasegawa 2012).

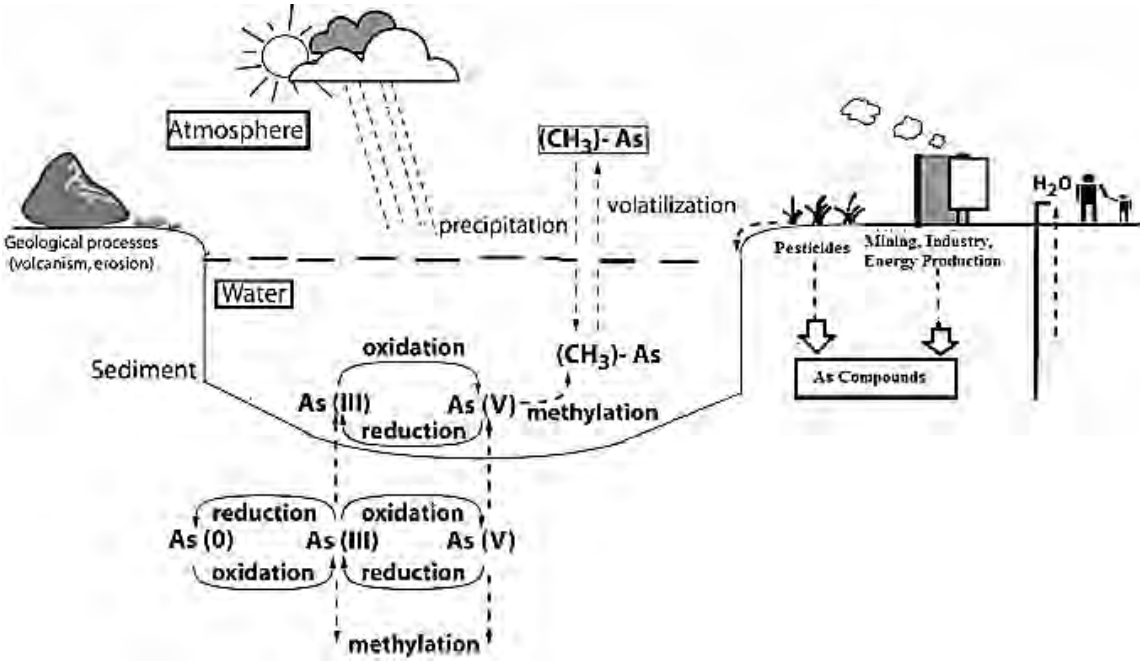


Figure 1 Global arsenic geocycle and effects from anthropogenic sources (Adapted from Sultana *et al.* 2015, after Mukhopadhyay *et al.* 2002).

Natural geological sources are one of the most significant causes of arsenic contaminated groundwaters around the world (Safiuddin and Karim 2001; Sharma and Sohn 2009; Bundschuh *et al.* 2012; Rodríguez-Lado *et al.* 2013; Alonso *et al.* 2014). Arsenic-containing bedrock formations are common in Bangladesh (India), and regions of China, where many cases of endemic contamination by arsenic are known (Garelick *et al.* 2009). Arsenic has been also documented as a significant water-quality problem in the Pampean Plain (Argentina), particularly in aquifers from Córdoba, La Pampa, Santa Fé and Buenos Aires Provinces (Nicolli *et al.* 1989; Smedley *et al.* 2002; Fiorentino *et al.* 2009), but also in other regions of this country (Bundschuh *et al.* 2004; Shaw *et al.* 2010). Moreover, several parts of the world have been affected by arsenic poisoning in soils, sediments and water due to past and recent mining activities (Smedley and Kinniburgh 2002; Wang and Mulligan 2006; Inam *et al.* 2011; Battogtokh *et al.* 2013). In fact, arsenic contamination of surface waters, especially in rivers (Table 1), is frequently driven by mining-related activities (Fig. 1). In particular, it has been reported that arsenic is mobilized during gold mining activities, because gold- and arsenic-bearing minerals coexist (Garelick *et al.* 2009). These anthropogenic activities may cause strong environmental disasters, as the one that occurred recently in Rio Doce River (Brazil), where illegal levels of arsenic and mercury have polluted the river with toxic mud in the days after a dam burst at an iron ore mine (The Guardian, 2015 November 26). Other arsenic anthropogenic inputs include indiscriminate use of certain pesticides and herbicides, as those reported in Australia, New Zealand and the US; as well as of wood preserving arsenicals, as in Europe and North America (Garelick *et al.* 2009; Rahman and Hasegawa 2012).

Table1 Mean arsenic concentration and ranges (numbers in parenthesis) (µg As L^{-1}) in river waters as reviewed by Smedley and Kinniburgh (2002).

Baseline	Polluted European rivers	Geothermal influenced	Mining influenced	High-As ground water influenced
0.83 (0.13-2.10)	24.75 (4.50-45)	38 (0.20-370)	137 (<0.20-7900)	425 (7-21800)

1.2 Arsenic speciation in freshwater ecosystems

Arsenic may occur in the environment in four oxidation states: +V (arsenate), +III (arsenite), 0 (arsenic) and -III (arsine). In natural waters it is mostly found in inorganic form (iAs), as oxyanions of pentavalent arsenate (As^{V}) and of trivalent arsenite (As^{III}) (Oremland and Stolz 2003; Sharma and Sohn 2009; Rahman *et al.* 2012). Quantification of arsenic species in water may be a difficult task since changes in the distribution of arsenic species may occur rapidly after sampling. In fact, As^{III} is easily oxidized to As^{V}, what would result in questionable speciation data (Hall *et al.* 1999; Francesconi and Kuehnelt 2004; Watts *et al.* 2010).

Concentrations and relative proportions of arsenic species vary according to changes in input sources, redox conditions and biological activity (Smedley and Kinniburgh 2002). Usually, arsenate (As^{V}) is the thermodynamically stable state in oxic waters, while arsenite (As^{III}) is predominant in anoxic and reduced environments (Smedley and Kinniburgh 2002; Rahman *et al.* 2012). Consequently, in lake and river waters (as well as in oxic seawater), As^{V} is generally the dominant species, whereas high relative proportions of As^{III} have been found in river stretches close to inputs of As^{III}-dominated industrial effluents and in waters with a component of geothermal water (Smedley and Kinniburgh 2002).

Redox potential (Eh) and pH are generally considered the most important factors controlling arsenic speciation (Smedley and Kinniburgh 2001), but the presence of As^{III} may be maintained in oxic waters by biological reduction of As^{V} (Smedley and Kinniburgh 2002). In fact, autotrophic and heterotrophic communities play a key role on the arsenic biogeochemistry (speciation, distribution and cycling) in the aquatic systems (Fig. 2), since they incorporate the dominant iAs and may convert it to other arsenic forms such as the organic methylarsenicals (Met-As, see sub-section 2.3.3) and/or higher order organoarsenicals (orgAs) like arsenosugars (Oremland and Stolz 2003; Rahman *et al.* 2012). Their biological activities may strongly influence the speciation and bioavailability of arsenic in water and sediments (Oremland and Stolz 2005), participating actively in its environmental cycle (Páez-Espino *et al.* 2009). These transformation reactions have a big impact on the environmental behavior of As, since the different chemical forms of this element exhibit different mobility (methyl As^{III} >> methyl As^{V} > As^{III} > As^{V}), and toxicity to higher organisms (methyl As^{III} > As^{III} > As^{V} > methyl As^{V}) (Huang 2014).

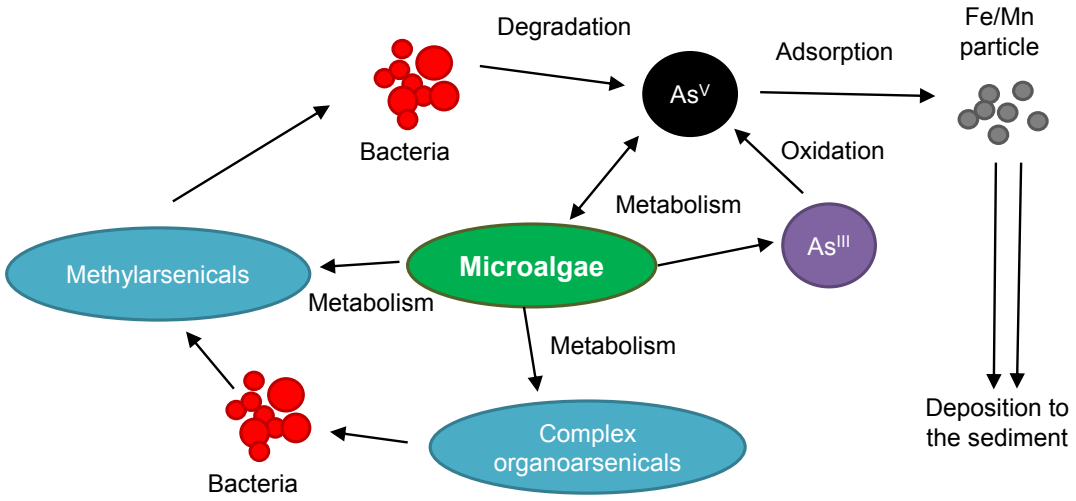


Figure 2 Main roles that microalgae and bacteria play in arsenic speciation and cycling in the aquatic systems. Adapted from Rahman *et al.* (2012).

Interestingly, the presence of phosphate (PO_4^{3-}) (in the medium and in the cell) can mediate the arsenic uptake and speciation in microorganisms (Levy *et al.* 2005; Wang *et al.* 2013). A simple model of arsenic speciation by algae under different phosphorus (P) conditions was suggested by Hellweger *et al.* (2003). The model predicts higher phosphate uptake and As^{III} excretion under non-P-limited than in P-limited conditions (Fig. 3).

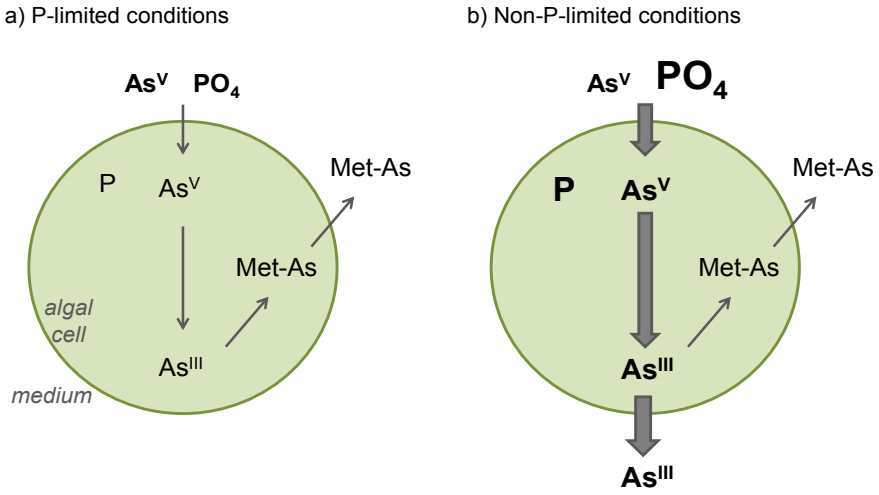


Figure 3 Diagram of arsenic speciation model by algae. Under P-limited conditions, algae take up As^V, reduce it to As^{III}, methylate it to Met-As, and then excrete it. However, under non-P-limited conditions, which exist in the early stages of blooms, algae upregulate their phosphate transport system to take up excess P. Large quantities of As^V are also taken up by the phosphate transport system at this time. Within the cell, the reduction of As^V to As^{III} is fast, but methylation is slower, causing As^{III} to build up in the cell. Intracellular As^{III} is then excreted, causing the increase in extracellular As^{III}. Size of text corresponds to species concentrations; size of arrows corresponds to rates. Adapted from Hellweger *et al.* (2003).

1.3 Arsenic in sediments and sediment-water interactions

Sediments are considered the ultimate sink for most natural and anthropogenic pollutants in the aquatic environment (Chen and Lin 2004). In freshwater systems, arsenic is predominantly bound to sediments, which may contain high amounts of this element (Brannon and Patrick 1987), especially in mining areas, where the concentration of arsenic can reach up to hundreds or thousands of mg kg^{-1} sediment (Smedley and Kinniburgh 2002). Arsenic may be incorporated into sediments as arsenic enriched particles eroded from weathered rock or soil and as colloids carrying adsorbed arsenic, which settle under low energy conditions, and as soluble arsenic that is retained by the inorganic, organic and biotic components of the sediments.

Arsenic distribution between the water column and the sediment is controlled by several physico-chemical and biological processes, such as flocculation/peptization, precipitation/solubilization, sorption/desorption, oxidation/reduction, penetration in the crystal structure of minerals and biological mobilization and immobilization (Matera and Le Hécho 2001; Sahuquillo *et al.* 2002). Changes caused directly by microorganisms in arsenic speciation, mobilization and toxicity are also relevant and will be explained in the next sub-section 2. Thus, in the present section, we will focus on the abiotic processes that cause arsenic mobility/retention in the environment, although most of them are indirectly driven by the activity of microorganisms (Huang 2014).

Precipitation may cause, under specific circumstances, the removal of As^{V} and As^{III} from solution. The topic has been extensively revised by Mandal and Suzuki (2002), Smedley and Kinninburgh (2002) and Drahota *et al.* (2009). Arsenate, similarly to phosphate, tends to precipitate with multivalent cations, such as aluminium and ferric iron under acidic conditions, and calcium and magnesium under alkaline conditions (Menció *et al*. 2016). Arsenate may also replace SO_4^{2-} or PO_4^{3-} in minerals, due to similar size and charge characteristics (Smedley and Kinniburgh 2002). In contrast, the solubility of arsenite is often controlled by sulfide precipitates, particularly where sulfidogenesis occurs under anoxic conditions, limiting As^{III} concentrations in extremely reducing environments (Moore *et al.* 1988; Oremland *et al.* 2002). Notwithstanding, although As^{III} and As^{V} can form insoluble compounds, precipitation is not usually a relevant arsenic removal mechanism, because the concentration of ions in the pore waters is generally too low to achieve the solubility product of the precipitates.

Sorption, here defined as any removal of a compound from solution to a solid phase (Sposito 2008), is considered the main mechanism for arsenic retention in freshwater sediments and responsible for the relatively low concentrations of arsenic found in most natural waters (Smedley and Kinniburgh 2002). This is the case of the Anllóns River (NW Spain), where concentrations up to 270 mg As^{V} kg^{-1} are found in sediments, whereas arsenic concentration is usually almost no detectable in river water (see the sub-section 3 and also ***Chapter 3*** for more details).

Sorption mechanisms include inner-sphere and outer-sphere surface complexation. The former, characterized by the formation of coordinated covalent bonds, is more stable than the latter, due to electrostatic interactions. The formation of inner-sphere complexes is the main but not the only mechanism for the sorption of As^{V} and As^{III} (Grossl *et al.* 1997; Manning *et al.* 1998; Arai *et al.* 2005; Catalano *et al.* 2008; Cheng *et al.* 2009). In recent years, studies based on arsenic sorption onto sediments were reported by Mandal *et al.* (2002), Rubinos *et al.* (2003), Bostick *et al.* (2004), Stollenwerk *et al.* (2007), and Borgnino *et al.* (2012). Arsenic adsorption capacity has been related to the content of metal oxides, particularly of Fe, Al and Mn (De Vitre *et al.* 1991; Sullivan *et al.* 1996; Smedley and Kinniburgh 2002) and to the clay content of the sediments (Smedley and Kinninburgh 2002). Iron oxides are probably the most important adsorbents because of their greater abundance and strong binding affinity, and their tendency to form coatings on the surface of the sediment particles (Smedley and Kinninburg 2002). Aluminium hydroxides and aluminosilicate clay minerals also retain appreciable amounts of arsenic, with a strong preference for As^{V} relative to As^{III} (Xu *et al.* 1988; Manning and Goldberg 1997a; Manning and Goldberg 1997b; Smith *et al.* 1998). In general, As^{V} binds extensively and strongly to most mineral constituents of the sediments, while As^{III} exhibits a limited binding, with the exception of iron (hydr)oxides for which As^{III} presents high affinity (Dixit and Hering 2003). This limited sorption makes As^{III} a more mobile oxyanion (Smedley and Kinniburgh 2002; Oremland and Stolz 2003).

Although less relevant than the aforementioned minerals, other sediment components contribute to arsenic sorption. Thus, for instance, the extent of arsenic sorption to, and coprecipitation on, carbonate minerals is still unknown but if it behaves as phosphate, it is likely to be strongly retained by these minerals (Millero *et al.* 2001). The presence of carbonates depends on the lithology of the basin, and these minerals are almost absent in siliceous regions where their effect on arsenic adsorption is negligible. Arsenic may also bind to organic matter in sediments; As^{V} adsorbs onto solid phase humic acids more extensively than As^{III}, with amine (NH_2) groups suspected as the primary functional group responsible for arsenic retention (Thanabalsingam and Pickering 1986). Biosorption of inorganic and organic arsenic species on surfaces of microbial cells living in the interface water-sediments has also been extensively demonstrated (Huang 2014); sorption of arsenic on bacterial cells is a pH dependent electrostatic interaction involving hydroxyl, amide and amino groups on the surface of the microorganisms (Yan *et al.* 2010; Prasad *et al.* 2011; Giri *et al.* 2013).

Arsenic can occur in the sediment–water systems in various chemical forms differing in mobility and toxicity. It is for this reason that total arsenic concentrations give a poor indication about its mobility, availability and potential risks for living organisms, and hence the determination of arsenic speciation is of great interest. Various approximations have been used for the study of arsenic speciation; the two most common are single extractions and chemical fractionations. With respect to single extractions, various procedures have been used to estimate the mobile fraction, such as leaching with deionized water, as described in the German

standard procedure DIN 38414–S4 (1984), or extraction with an acetic acid solution, as in the toxicity characteristic leaching procedure (TCLP) (US EPA 1992), which estimates the leaching potential of arsenic and its effect on the survival of microorganisms (*Aliivibrio fischeri*). Other tests aim at evaluating the available concentration of the element for a targeted group of organisms, such as the extraction in 1 M HCl, for the estimation of the arsenic available fraction to plants (Snape *et al.* 2004; Moalla *et al.* 2006), and the use of a physiologically based extraction test (PBET) (Ruby *et al.* 1996) for the estimation of bioavailability to superior animals.

In turn, fractionation procedures are based on the extraction and quantification of operationally defined fractions of different mobility. A number of chemical extraction schemes have been devised which attempt to allocate elements to particular solid phases, but few of them are specifically designed for the speciation of solid phase arsenic. Among them, the procedure of Lombi *et al.* (2000) has been widely used for arsenic speciation; it consists of a 6-step sequential extraction, aiming at solubilizing various forms of decreasing mobility: exchangeable, specifically sorbed, associated to Al and organic matter, bound to amorphous Fe oxides, bound to crystalline Fe oxides, and the residual and less mobile phase. In turn, Keon *et al.* (2001) have described a 8-step extraction scheme that attempted to partition sediment arsenic into ionically-bound; strongly adsorbed; acid extractable volatile sulfides, carbonates, Mn oxides and very amorphous Fe oxyhydroxides; oxalate extractable amorphous Fe oxyhydroxides; crystalline Fe oxyhydroxides; arsenic oxides and silicates; pyrite and amorphous As_2S_3; and orpiment and other recalcitrant minerals. Although the selective extraction schemes are not perfect and universally applicable, they reflect the variable nature of arsenic in the solid phase and therefore its potential behaviour or availability.

Sediments are increasingly recognized as both carriers and possible sources of contaminants in aquatic systems (Förstner and Salomons 1991). They play an important role in maintaining water quality by removing contaminants from the water column, acting as sinks for contaminants. However, subsequent remobilization of contaminants from the sediment can maintain high concentrations of dissolved contaminants long after the initial source has been removed (Linge 2008), so that the sediment itself can act as a contaminant source. Although freshwater sediments usually act as sinks for arsenic in river systems, changes in environmental conditions (Eh, pH, water composition) may promote arsenic release from the solid phase to the water column. In this case, arsenic enriched sediments may act as ‘‘chemical time bombs’’ (CTB) (Cappuyns *et al.* 2006), as they may release arsenic under certain favorable circumstances, posing a risk to aquatic life and human health (Anawar *et al.* 2004; Fendorf *et al.* 2008). Processes leading to arsenic release from solid phases can be broadly grouped into four categories: (1) alkalinity, causing desorption (or limited sorption), (2) ion displacement, (3) reducing conditions, leading to reduction of arsenate to the more mobile arsenite, and (4) mineral dissolution, particularly reductive dissolution of Fe and Mn (hydr)oxides (Fendorf 2008). These four categories are explained below.

High pH (>8.5) promotes arsenic mobility by favoring arsenic desorption and preventing it from being adsorbed. The effect of alkaline conditions in the increased mobility of arsenic has been demonstrated under laboratory conditions by Rubinos *et al.* (2010), who have observed that the percentage of arsenic released from sediments was 10 to 45 times higher at pH 10 than at pH 4; moreover, leaching of arsenic at alkaline pH was accompanied by the release of Fe, Al and organic matter that are potentially important sinks for arsenic in sediments.

Competitive ion displacement may cause arsenic release to the aqueous phase. Phosphate, carbonate and bicarbonate ions are commonly reported as competing ions and may inhibit arsenic adsorption or increase arsenic leaching from mineral surfaces (Sharma and Sohn 2009). Dissolved silicate (Luxton *et al.* 2008) and organic matter (Xu *et al.* 1991; Grafe *et al.* 2001; 2002; Redman *et al.* 2002) can also competitively limit arsenic adsorption and promote its desorption. Other anions, such as Cl^-, SO_4^{2-}, and NO_3^- have less impact on arsenic retention, yet these ions contribute to ionic strength that is important in arsenic desorption (Gupta and Chen 1978; Smith *et al.* 1998; Rubinos *et al.* 2011a).

Mobilization of arsenic by phosphates is of particular concern (Manning and Goldberg 1996; Reynolds *et al.* 1999; Dixit and Hering 2003; Fendorf *et al.* 2008), since phosphate and arsenate strongly compete for sorption sites, thereby making arsenate more mobile under conditions of phosphate abundance (Oremland *et al.* 2002). Competition is due to the similarities of phosphorus and As^V, as both form oxyanions, have similar p*Ka* values and exhibit similar aqueous speciation as a function of pH. In the literature, the mobilization of arsenic by P from sediments has been widely reported by Kaplan and Knox (2004), Bauer and Blodau (2006), Stollenwerk *et al.* (2007), Rubinos *et al.* (2010, 2011b), among others. The introduction of waters containing high concentrations of phosphate can therefore displace arsenic from sorption sites through competitive ligand-exchange reactions (Peryea 1991; Violante and Pigna 2002; Pigna *et al.* 2006) and regions where fertilizer or pesticide runoff and leaching occur are specifically at risk for this mobilization pathway (Peryea and Kammerack 1997; Jain and Loeppert 2000).

In what concerns Eh conditions, numerous studies have demonstrated the release of arsenic under reducing conditions in the sediments (Aggett and O'Brien 1985; Moore *et al.* 1988; Azcue and Nriagu 1995; Widerlund and Ingri 1995). Although iron reduction and dissolution have been associated with arsenic release (de Vitre *et al.* 1991; Guo *et al.* 1997), arsenic reduction may also be influential, because arsenite forms more labile complexes on ferric (hydr)oxides (Bose and Sharma 2002).

Microorganisms play a key role in some of these processes of arsenic release from sediments, participating mainly in the reduction of As^V to As^{III}, and in the dissolution of Fe and Mn oxides that act as arsenic carriers (Fig. 4). In fact, studies on natural systems clearly revealed that microorganisms are major players to drive the arsenic cycle in the aquatic surface environments (Huang 2014). The role of the microorganisms constituting biofilms on arsenic

biogeochemistry in the sediment-water interface of freshwater systems will be discussed in sub-section 2.

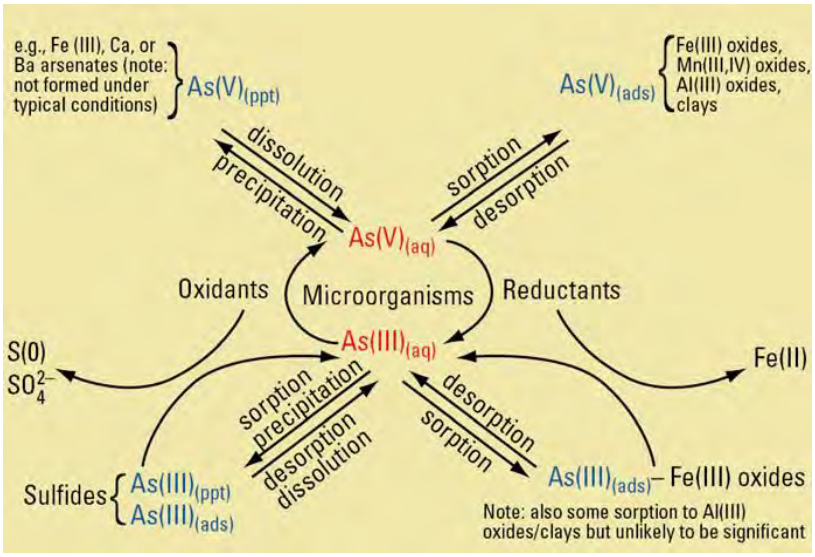


Figure 4 Possible processes in biogeochemical cycling of arsenic (Reisinger *et al.* 2005). As(V): arsenate; As(III): arsenite; ppt: precipitated; ads: adsorbed; aq: aquatic.

2. THE ROLE OF BIOFILMS ON ARSENIC BIOGEOCHEMISTRY

The fate of arsenic released into the environment by natural processes or anthropogenic activities is determined by a complex interplay among processes of arsenic mobilization, sequestration and transformation, most of which are directly or indirectly driven by the activity of microorganisms (Huang 2014). For many years, the study of arsenic cycling was focused on chemical and physical processes but, nowadays, there is no doubt about the involvement of microorganisms on it (Oremland and Stolz 2003, 2005). In fact, it is generally considered that arsenic transformation in the environment is mostly a biotic process, being the abiotic transformation substantially slower and less important (Huang 2014).

2.1 Biofilms in freshwater systems

Microorganisms play a key role on the arsenic biogeochemistry (Rahman and Hassler 2014), especially microalgae and prokaryotes. In freshwaters, these microorganisms may occur as complex and structured benthic communities, living closely together in a matrix composed of extracellular polymeric substances (EPS matrix). These structured benthic communities receive the name of biofilm, phytobenthos or periphyton, in which green algae, diatoms, and cyanobacteria form the photoautotrophic component, while bacteria, fungi and protozoa compose principally the heterotrophic one (Romaní 2010; Mora-Gómez *et al.* 2016; Neury-Ormanni *et al.* 2016).

The mechanical stability of biofilms is provided by the EPS matrix, mediating their adhesion to surfaces and immobilizing biofilm cells (Decho 2000; Gerbersdorf *et al.* 2008;

Wingender and Flemming 2011). The EPS can be produced by bacteria, but some of the most abundant EPS producers are microalgae (in particular, diatoms, the microscopic, unicellular brown algae). Owing to the stickiness of the matrix, particles and nanoparticles from the water phase may be trapped and accumulated (Flemming and Wingender 2010; Huang 2014). Cations, anions and apolar compounds may also be adhered and adsorbed by physico-chemical mechanisms. Consequently, biofilms integrate the environmental effects of water chemistry, being the reason why, along with the physical and geomorphological characteristics of rivers and lakes, they have been widely applied in biomonitoring, being diatoms extensively used as reliable environmental indicators (Morin *et al.* 2016).

Aquatic biofilms have a large variability in structure and composition. Their high complexity is related to the type of substratum where they develop and the environment in which they are living (Karatan and Watnick 2009). In natural environments, biofilm grows upon inert substrata such as sand or sediment, rocks and cobbles; also, on dead organic substrata such as wood, leaf litter or particulate organic matter; and living plants such as aquatic macrophytes and macroalgae. Microorganisms attached to the particles of sandy sediments are referred to as epipsammon or epipsammic biofilms (Fig. 5.a). They are more heterotrophic (with higher contributions of bacteria and fungi) than the biofilm developed on rocks, and play a key role in organic matter decomposition, especially in rivers (Pusch *et al.* 1998; Romaní and Sabater 2001). In aquatic environments, sediments usually show a profile zonation in depth if they are sufficiently thick, changing from an oxidized zone in the surface sediment to a reduced zone in deeper layers, creating anoxic zones (Boulton *et al.* 1998). In this regard, surface sediments support heterotrophic communities that include more opportunistic species in comparison with other biofilms such as those developed on rock surfaces (Mora-Gómez *et al.* 2016). Biofilms attached to natural inorganic-hard surfaces as rock, gravel and cobble, are referred to as epilithic biofilms, epilithon or, more generally, periphyton (Mora-Gómez *et al.* 2016; Fig. 5.b). They develop a more complex structure with a higher microalgal biomass and they are more independent of seasonal fluctuations, compared to epipsammic biofilms (Romaní and Sabater 2001; Graba *et al.* 2013).

Energy transformations within biofilms include the conversion of light into chemical energy by microalgal photosynthesis, the adsorption and microbial uptake of heterotrophic carbon (C), and internal transfers due to extracellular release and cell lysis, leading both autotrophs and heterotrophs to be benefited from the internal fluxes of this highly symbiotic association (Allan and Castillo 2007). Although autotrophic–heterotrophic relationships may occur in most aquatic biofilms, microalgal–bacterial interactions have been mainly described for epilithic biofilms, in which the structural stability and close spatial relationship between heterotrophic bacteria and microalgae favors the bacterial use of fresh labile organic compounds released by microalgae (Wetzel 1993; Sobczak 1996; Romaní and Sabater 1999, 2000), affecting the whole biofilm metabolism. In addition, when a thick biofilm is developed in environments which are rich in nutrients, an anoxic layer may exist at the biofilm bottom and, consequently, anaerobic bacteria may be present (Schramm *et al.* 1999; Flemming *et al.* 2016).

This micro-spatial variation contributes to the intra-site differences observed in different communities of microorganisms (Anderson-Glena *et al.* 2008). Actually, biofilms allow the coexistence of microniches of different physiological requirements, enabling the simultaneous, but spatially separated occurrence of opposing redox processes in the same biofilm environment (Huang 2014). Moreover, biofilms are complex sets of communities that may experience large diel variations in oxygen tension as a response to daytime net photosynthesis and night-time net respiration (Kulp *et al.* 2004). Therefore, we may consider biofilm as a community and also as an environment for microorganisms. Such an environment, it may provide niches for As^{V} reducers and As^{III} oxidizers (Kulp *et al.* 2004), so that there might be simultaneous arsenic oxidation and reduction in biofilms (Huang 2014).

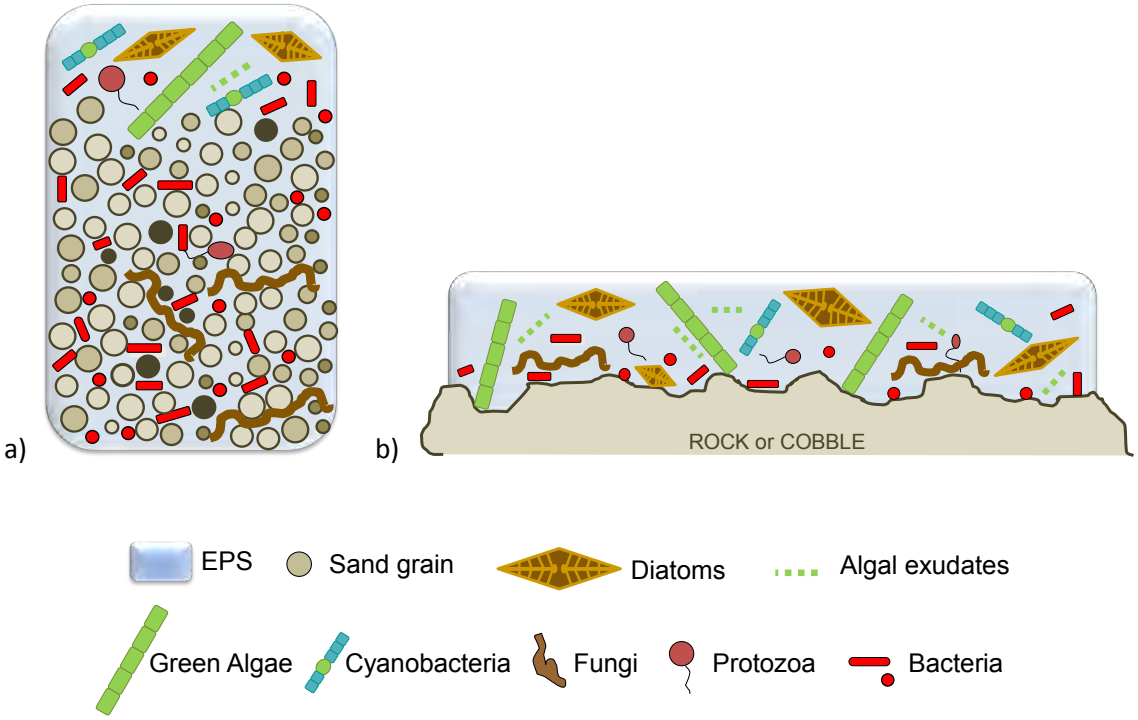


Figure 5 Freshwater-biofilm types regarding to their growth on (a) sand, named epipsammon, or on (b) inorganic hard substrates like rock, named epilithon or periphyton EPS: extracellular polymeric substances. Adapted from Mora-Gómez *et al.* (2016).

From now on, in the text we will consider as “microalgae” all aerobic-photoautotrophic organisms including eukaryotes and cyanobacteria; while “prokaryotes” will be referred to archaea and bacteria, except cyanobacteria, what includes all heterotrophic and chemolithoautotrophic microorganisms, as well as anaerobic-photoautotrophic bacteria such as purple bacteria.

2.2 Arsenic biosorption, uptake and bioaccumulation

Several types of functional groups such as carboxyl, hydroxyl, carbonyl, sulfhydryl and amino groups on the cell surface are responsible for superficial adsorption of metals and

metalloids, including arsenic (Wang *et al.* 2015). Sorption of arsenic species such as As^{III} and As^{V} has been identified as a pH-dependent electrostatic interaction (Huang 2014). Adsorption is a relatively rapid and reversible process, which plays an important role in arsenic detoxification in a wide variety of bacteria and, especially, in microalgae species. Microalgae may adsorb up to 60 % of total arsenic amount in water, being the reason why they are highly used for bioremediation (Wang *et al.* 2015).

In what concerns uptake, microalgae and prokaryotes have not developed any dedicated arsenic uptake system (Páez-Espino *et al.* 2009). Instead, arsenic enters cells via existing transport systems, such as phosphate transport for As^{V} and aquaglyceroporins (AQP) for As^{III}. The chemical similarity between PO_4^{3-} and AsO_4^{3-} suggests that these ions may show competitive behavior with regard to the phosphate uptake system (Wang *et al.* 2015). No clear defined As^{V} transport system is still described, particularly in algal uptake, but it was hypothesized that different mechanisms are involved as a function of P availability (i.e., in cyanobacteria, by Guo *et al.* 2011). For instance, it was observed that under phosphate-deprived condition, a large quantity of arsenate may be taken up by starved cells because of phosphate deficiency (Guo et al 2011); while increasing phosphate in the medium may lead to decrease the uptake of arsenate (and resulting toxicity) by freshwater microalgae (Levy *et al.* 2005; Guo *et al.* 2011). Concerning the As^{III} uptake, membrane hexose permeases and AQP were detected to be the transporting systems at physiological pH since, under that conditions, As^{III} is present as non-charged $As(OH)_3$ rather than as its oxianionic form (Páez-Espino *et al.* 2009; Wang *et al.* 2015; Escudero-Lourdes 2016).

Once inside the cell, arsenic may be metabolized, what involves different arsenic transformations. Arsenic species may then be excreted outside the cells or rested inside bioaccumulated (Huang 2014). In the food web, microalgae may accumulate arsenic more efficiently than higher organisms (Wang *et al.* 2015).

2.3 Arsenic biospeciation

Despite the high toxicity of the two inorganic forms of arsenic, As^{V} and As^{III}, after their adsorption and biosorption some microorganisms can transform them (biospeciation) and even gain energy from this process. In general, the biochemical transformation of arsenic includes oxidation of As^{III} to As^{V}, reduction of As^{V} to As^{III}, As^{III} accumulation inside cells, arsenic methylation and further transformation to more complex organic forms such as arsenosugars or arsenolipids, as well as demethylation and arsenic excretion from cells (Fig. 6). Many abiotic factors affect the arsenic metabolism, such as the composition of growth medium, arsenic species and levels, pH, temperature, Eh, exposure duration, light intensity and photoperiod (Wang *et al.* 2015).

Arsenic biochemistry was highly reviewed by several authors for aerobic and anaerobic prokaryotes and for microalgae, separately. Here, we studied together both kinds of microorganisms, prokaryotes and microalgae, to better understand how they may interact during the As-biospeciation in biofilms.

2.3.1 Arsenite oxidation

Oxidation of As^{III} to As^{V} by microorganisms has an important impact, since it reduces arsenic mobility in the environment, due to the usually higher affinity of As^{V} than that of As^{III} for mineral surfaces (Smedley and Kinniburgh 2002; Huang 2014). This is a detoxification process because As^{V} is less toxic than As^{III} to prokaryotes and higher organisms such as fish and humans (Páez-Espino *et al.* 2009; Nagvenkar and Ramaiah 2010; Rahman and Hassler 2014; Wang *et al.* 2015). Microbial oxidation is faster than chemical oxidation of As^{III} to As^{V} (Stolz *et al.* 2006; Hudson-Edwards and Santini 2013). In microalgae (see Fig. 6.a), this biotransformation has received little attention, but it was reported in the cell surface of some acidophilic red algae and some cyanobacteria, especially under increased P levels in the medium (Qin *et al.* 2009; Zhang *et al.* 2013a). Also, arsenite oxidation is usually observed on the cell's outer membrane of archaea and bacteria, including anaerobic-photosynthetic purple bacteria (Kulp *et al.* 2008), being especially studied in chemolithoautotrophic arsenite oxidizers (CAOs) and heterotrophic arsenite oxidizers (HAOs) (Fig. 6.b). All known CAOs are bacteria (Amend *et al.* 2014). Specifically, CAOs obtain energy from the oxidation of As^{III} to As^{V} in combination with O_2 or nitrate, under aerobic and anoxic conditions respectively, while obtaining inorganic C from the fixation of CO_2 (Oremland and Stolz 2005; Páez-Espino *et al.* 2009; Hudson-Edwards and Santini 2013; Huang 2014; Rahman and Hassler 2014). On the other hand, HAOs also convert As^{III} into the less toxic form As^{V} (detoxification reaction) while respiring oxygen, but, in contrast to CAOs, they cannot fix CO_2 and instead they use organic C for making cell material; that is, they need organic matter for cell growth (Oremland and Stolz 2003, 2005; Hudson-Edwards and Santini 2013). Under aerobic conditions, in both HAOs and CAOs the key enzyme is the arsenite oxidase, recently named Aio and formerly referred to in the literature as Aro, Aox or Aso. The As^{III} which enters the periplasm is oxidized to As^{V} by the Aio and then the electrons are transferred to other proteins involved in the electron transport chain which results in the production of ATP and the reduction of oxygen to water. Conversely, the anaerobic arsenite oxidation by CAOs (using nitrate instead oxygen as electron acceptor) is via Arx enzyme, which is less well-characterized (Hudson-Edwards and Santini 2013; Amend *et al.* 2014).

2.3.2 Arsenate reduction

Reduction of As^{V} to As^{III} leads to an increase in arsenic mobility and toxicity in the natural environment (Wang *et al.* 2015), and it has been commonly observed in bacteria, archaea and microalgae (Oremland and Stolz 2003; Rahman and Hassel 2014), both extra- (anaerobic) and intracellularly (aerobic). Extracellularly, aqueous or solid-phase arsenate can be used as the ultimate electron acceptor during anaerobic respiration (Páez-Espino *et al.* 2009) by the denominated dissimilatory arsenate-reducing or arsenate-respiring prokaryotes (DARPs) through oxidation of organic electron donors (Oremland and Stolz 2005; Huang 2014) (see Fig. 6.b). These anaerobic prokaryotes (mainly bacteria and hyperthermophilic archaea) are opportunists capable of respiratory growth on a wide diversity of electron donors (Kulp *et al.* 2004; Oremland and Stolz 2005), conserving metabolic energy from the reduction of As^{V}

(Amend *et al.* 2014). For instance, in biofilms, algal exudates are an important source of organic matter and provide an abundant supply of electron donors to these microorganisms (Kulp *et al.* 2004). This anaerobic respiration usually occurs in subsurface water aquifers, where important influx of organic materials take place; however, even microbial mats themselves may promote microbial respiration and the onset of anoxia (Oremland and Stolz 2003), as in the bottom layer of biofilms (Fig. 6.c). The DARPs then respire adsorbed As^{V}, causing dissolution of adsorbent minerals (e.g. ferrihydrite) and resulting in the production and release of As^{III} into the aqueous phase (Oremland and Stolz 2003). The As^{V} reductase of DARPs is named Arr, while the arsenate respiratory reductase-encoding gene is *arrA* (Bertin *et al.* 2011).

Intracellularly, the mechanisms of As^{V} reduction are through cell detoxification, since As^{V} is an analogue of phosphate and, therefore, a potent inhibitor of photophosphorylation and oxidative phosphorylation, key reactions of energy metabolism in organisms, impeding the synthesis of ATP (Oremland and Stolz 2005; Huang 2014; Wang *et al.* 2015). Microorganisms actively take up the toxic As^{V} through the PO_4^{3-} uptake system (Rahman and Hassler 2014) and can then expell it from the cell, after reducing it to As^{III} to facilitate its export (Oremland and Stolz 2005; Wang *et al.* 2015). Many studies support this biotransformation model in microalgae (Fig. 6.a) and aerobic prokaryotes (Fig. 6.b) (e.g. Levy *et al.* 2005; Hudson-Edwards and Santini 2013; Amend *et al.* 2014; Wang *et al.* 2015), being able to survive in aquatic habitats with raised As^{V} levels (Wang *et al.* 2015). These microorganisms that reduce As^{V} to As^{III} as a means of resistance are called arsenate-resistant microorganisms (ARMs, referred specially to prokaryotes; see Fig. 6.b), and do not gain energy from the process, but use it as a means of coping with high arsenic levels in their environment (Oremland and Stolz 2005). The best studied resistance mechanism is the Ars (arsenic resistance system). Prokaryotes, especially bacteria, may be resistant to As^{V} through the expression of three genes (*arsR, arsB* and *arsC*) in an *ars* operon, which encodes a cytoplasmic arsenate reductase (ArsC) enzyme and As^{III} efflux pump (ArsB) (Stolz *et al.* 2006; Patel *et al.* 2007; Wang *et al.* 2015): aqueous As^{V} that has entered to the cytoplasm is reduced to As^{III} through a process mediated by this ArsC and, the resulting As^{III}, which is far more soluble, is then generally pumped out of the cell by an active transport system (the ArsB protein), which requires energy through ATP hydrolysis (Oremland and Stolz 2005; Hudson-Edwards and Santini 2013; Amend *et al.* 2014). Similarly, in cyanobacteria it was identified an operon of three genes (*acr3*, *arsH* and *arsC*), encoding a cytoplasmic arsenate reductase (ArsC) enzyme and an As^{III} efflux pump, named Acr3 (López-Maury *et al.* 2003). It is suggesting that ArsH plays a role in the resistance to arsenic, specifically in the response to oxidative stress caused by arsenite (Hervás *et al.* 2012).

If, on the other hand, the aqueous As^{III} is the arsenic species that enters the cell, it can be pumped straight out via ArsB (Hudson-Edwards and Santini 2013). However, As^{III} may also stay sequestered inside the cell, bound to cysteine residues in enzymes (Páez-Espino *et al.* 2009) (see Fig. 6.b). Many researchers consider that As^{III} in microalgae is much more easily removed than As^{V} from the cells through the sequestration of stable complexes with glutathione

(GSH) or phytochelatins (PCs) into the vacuole, followed by excretion out of the cells as a detoxification process (Rahman and Hassler 2014; Wang *et al.* 2015).

2.3.3 Arsenic methylation

Arsenic methylation appears to be a detoxification process, since the obtained products may be generally less toxic than the inorganic species (Wang *et al.* 2015); and also a mobilization process, due to the lower adsorption affinity of methylated arsenic than iAs species (Huang 2014). Methylarsenicals (Met-As) in natural waters are expected to be produced directly by aerobic and anaerobic microorganisms, including prokaryotes and microalgae, through reduction of As^{V} to As^{III} and subsequent methylation (Páez-Espino *et al.* 2009; Wang *et al.* 2015). The activities of these microorganisms are responsible for the seasonal variations of methylarsenic compounds in freshwaters (Rahman and Hasegawa 2012). Arsenic methylation by microalgae has been especially well documented (Fig. 6.a). However, the mechanisms of methylation are controversial (Huang 2014; Wang *et al.* 2015).

Inorganic arsenic species can be methylated to the organic As-species monomethylarsenate (MMA^{V}), also named monomethylarsonic acid ($MMAA^{V}$), and to dimethylarsenate (DMA^{V}) or dimethylarsinic acid ($DMAA^{V}$), the reaction being catalyzed by the arsenite methyltransferases (ArsM) (Qin *et al.* 2009; Amend *et al.* 2014; Wang *et al.*, 2015). In addition to MMA^{V} and DMA^{V}, As^{III} methylation products also include less toxic arsenic species such as trimethylarsine oxide (TMAO), detected in fluvial diatoms (Kaise *et al.* 1997), and the volatile and almost non-toxic trimethylearsine (TMA) (Mukhopadhyay *et al.* 2002; Wang *et al.* 2015). Biological production of TMA is minor, as shown by Prieto *et al.* (2016a) for epipsammic biofilms, and particularly in cyanobacteria (Yin *et al.* 2011). However, in recent years it has become apparent that methylation of As^{III} may not be necessarily a detoxification process, since metabolic species such as monomethylarsenite (MMA^{III}), also named methylarsonous acid ($MMAA^{III}$), and dimethylarsenite (DMA^{III}) or dimethylarsinous acid ($DMAA^{III}$) are more toxic than their As^{III} analogs, despite not being very stable and usually undetected in the microalgae or in the media (Wang *et al.* 2015). Environmentally, the trend in toxicity of arsenic species (most to least) for organisms is, in general: $MMA^{III} = DMA^{III} > As^{III} > As^{V} > MMA^{V} = DMA^{V} > TMAO^{V} = TMAO^{III} >$ TMA (Mukhopadhyay *et al.* 2002; Rahman and Hassler 2014; Wang *et al.* 2015). Some authors as Qin *et al.* (2009) have detected in bacteria that although MMA^{III} and DMA^{III} are more toxic than As^{III}, they do not accumulate in cells expressing *arsM.*

Different methylation pathways by microorganisms are proposed. For instance, a main route in microalgae may be $As^{V} \rightarrow As^{III} \rightarrow MMA^{V} \rightarrow MMA^{III} \rightarrow DMA^{V} \rightarrow DMA^{III}$ (Quin *et al.* 2009), or $As^{III} \rightarrow MMA^{III} \rightarrow DMA^{III} \rightarrow DMA^{V}$ (Zhang *et al.* 2013b). Aerobic and anaerobic methylation pathway(s) by prokaryotes would be similar to the microalgal ones (Cullen *et al.* 1994; Páez-Espino *et al.* 2009; Huang 2014); however, arsenic methylation by bacteria is focused in the production of gaseous arsines as the less toxic TMA (Fig. 6.b).

In summary, the function of biomethylation of toxic iAs is nowadays controversial (Rahman and Hassler 2014) and, whether biomethylation may be considered a detoxification

process or not depends on which Met-As species the microorganisms predominantly produce. This ability depends on the microorganism species and the nutritive status of the environment, as well as on the seasonal variables such as light and temperature (Rahman and Hassler 2014). Higher arsenic concentrations and/or longer exposure times may be other important factors causing the production and efflux of methylated arsenic species. Either way, it was found that microalgae are more likely to methylate arsenic under P-limited conditions (Hellweger *et al.* 2003; Levy *et al.* 2005; Wang *et al.* 2015), that is, under increased As^{V}/P ratios in the water column. It is also possible to find Met-As species in natural waters as a consequence of the breakdown of dead cells, excretory products and the degradation of arsenosugars or arsenolipids from decomposing cells (Oremland and Stolz 2003; Wang *et al.* 2015).

2.3.4 Synthesis of arsenosugars and arsenolipids

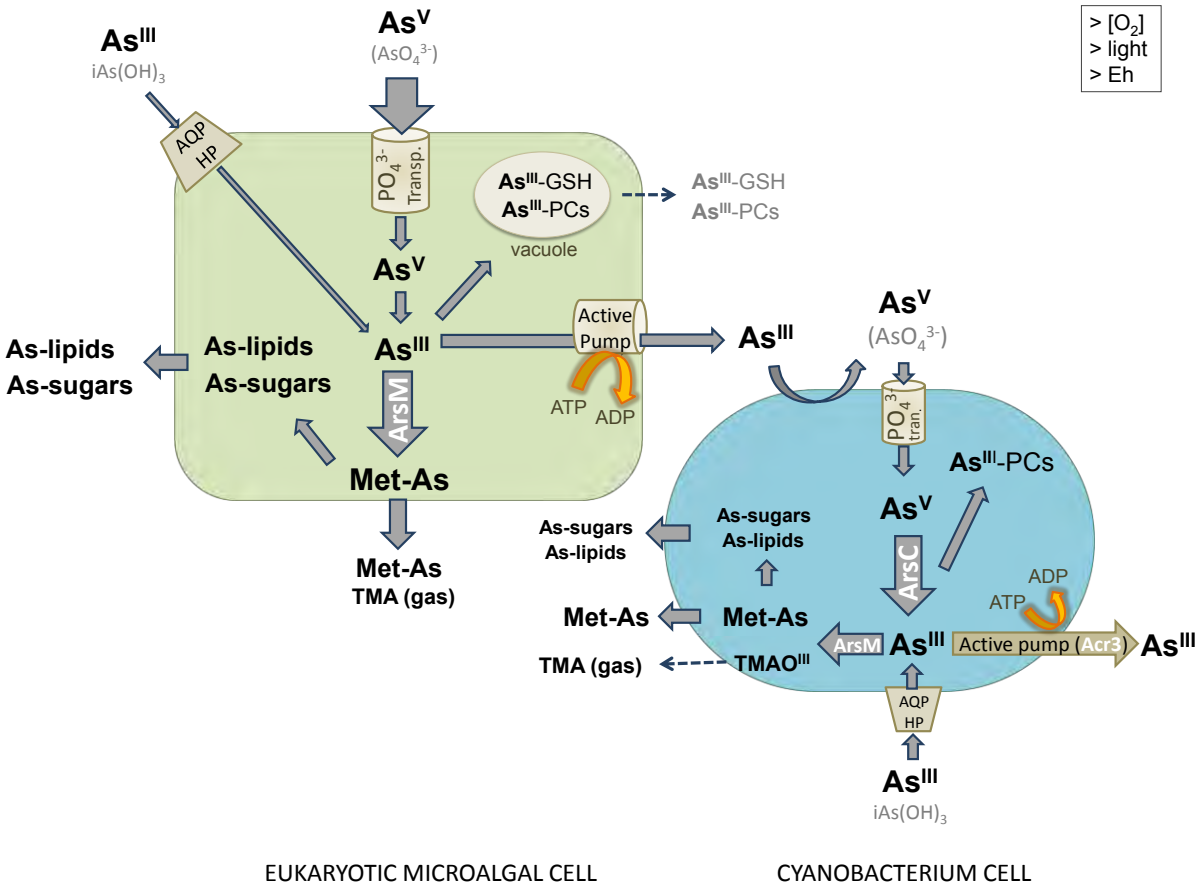
After methylation, microorganisms may also synthetize some organic arsenosugars (As-containing sugars) and arsenolipids (As-containing lipids). This biosynthesis was more studied in marine environments rather than in freshwaters, and the pathway seems to be unclear. Nonetheless, it was detected that some freshwater microalgae (Fig. 6.a) may biosynthesize arsenosugars after As^{V} reduction and posterior methylation (Miyashita *et al.* 2011; 2012). Arsenosugars have less toxicity than their inorganic counterparts and are of especial interest because they are widespread in many different aquatic organisms (Sharma and Sohn 2009). Arsenobetaine (As-Bet) is very commonly found in seafood and MMA^{V} and DMA^{V} are their most commonly reported degradation products formed upon cooking aquatic organisms (Sharma and Sohn 2009); however, little is known about As-Bet in freshwater organisms (Caumette *et al.* 2012).

The identity and toxicity of arsenolipids are largely unknown due to the high difficulties to isolate and analyze them compared to water-soluble arsenic species (Wang *et al.* 2015). Nonetheless, arsenolipid biosynthesis dependent on the catalysis by As^{III} methyltransferase (ArsM) was found, for instance, in a freshwater cyanobacterium, especially under low As^{V} concentrations (Xue *et al.* 2014). Moreover, it seems that some arsenolipids could be degraded to more toxic arsenic species in higher organisms (Meyer *et al.* 2014), what could contribute to increase arsenic toxicity in the environment, probably affecting the ecosystem functioning.

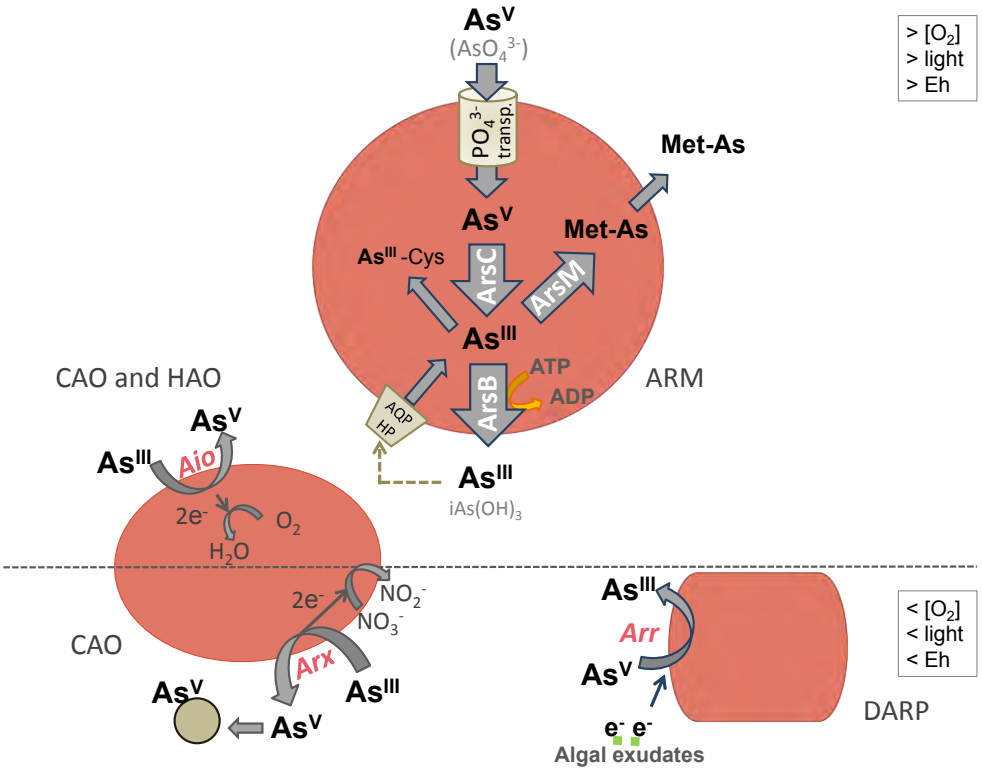
2.3.5 Demethylation

Demethylation of a methylarsenic molecule is the chemical process resulting in removal of a methyl group (CH_3). Although As-demethylation by microorganisms has been broadly evidenced in the natural environment, characterization of microbial demethylation and investigation of the involved microbial community is scarce (Huang 2014). It is expected that this biodemethylation may have important implications in the arsenic cycle and in the ecological status of aquatic systems, since it may increase the release of iAs species into the water and, consequently, increase its toxicity.

a)


> [O2]
> light
> Eh
AsIII
iAs(OH)3
AQP HP
AsV
(AsO4 3-)
PO4 3- Transp.
AsIII-GSH
AsIII-PCs
vacuole
AsIII-GSH
AsIII-PCs
AsV
As-lipids
As-sugars
As-lipids
As-sugars
AsIII
Active Pump
ATP
ADP
ArsM
Met-As
Met-As
TMA (gas)
AsIII
AsV
(AsO4 3-)
PO4 3- tran.
AsIII-PCs
AsV
As-sugars
As-lipids
ArsC
ADP
ATP
As-sugars
As-lipids
Met-As
Met-As
ArsM
AsIII
Active pump (Acr3)
AsIII
TMA (gas)
TMAOIII
AQP HP
AsIII
iAs(OH)3
EUKARYOTIC MICROALGAL CELL
CYANOBACTERIUM CELL

b)


> [O2]
> light
> Eh
AsV
(AsO4 3-)
PO4 3- transp.
Met-As
AsV
Met-As
AsIII -Cys
ArsC
ArsM
AsIII
ATP
ADP
ArsB
AQP HP
AsIII
iAs(OH)3
CAO and HAO
ARM
AsV
AsIII
Aio
2e-
O2
H2O
2e-
NO2-
NO3-
CAO
Arx
AsV
AsIII
AsV
AsIII
Arr
AsV
e- e-
Algal exudates
< [O2]
< light
< Eh
DARP

c)

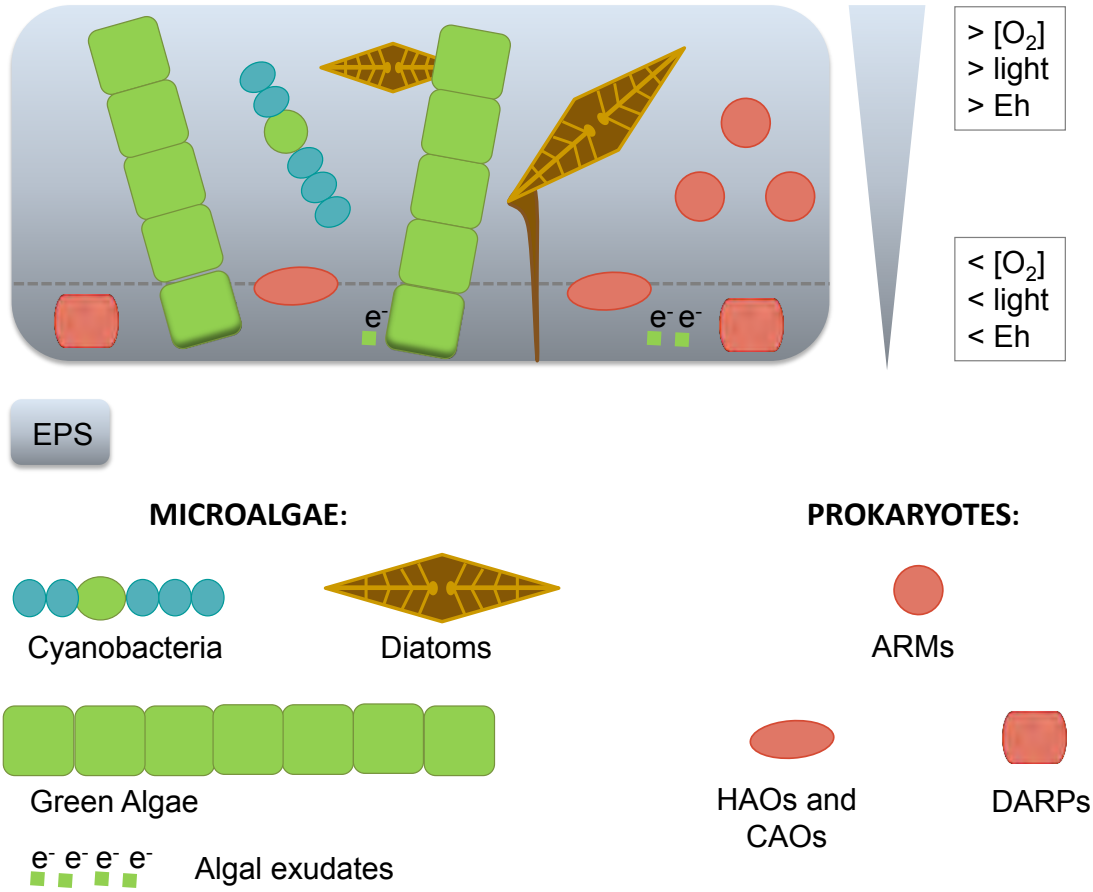


Figure 6 Biospeciation in freshwater biofilms: main speciation processes in (a) cyanobacterium and eukaryotic micoalgal cells (green algae and diatoms), and in (b) aerobic and anaerobic prokaryotes (arsenate-resistant microorganisms, ARMs; heterotrophic arsenite oxidizers, HAOs; chemolithoautotrophic arsenite oxidizers, CAOs; dissimilatory arsenate-respiring prokaryotes, DARPs). The biofilm redox profile zonation in depth and consequent location of microorganisms (c) is also illustrated. Transp.: transporters. AQP: aquaglyceroporins. HP: hexose permeases. GSH: glutathione. PCs: phytochelatins. Cys: cysteine residues in enzymes. See main text for details.

3. ARSENIC TOXICITY

3.1 Arsenic toxicity in microorganisms

The response of microorganisms to arsenic is known to result in various biological effects, including oxidative stress, DNA damage, alteration of exopolysaccharide synthesis and biofilm formation (Bertin *et al.* 2011). In addition, different arsenic species have different modes of toxic action to organisms (Rahman and Hassler 2014). Arsenite inhibits the production of glutathione, which protects cells against oxidative damage and represents the basic component of phytochelatins, important molecules for the detoxification of numerous metals in phytoplankton and plants (Rahman and Hassler 2014). Consequently, arsenite toxicity in the cell results in membrane degradation and cell death by producing reactive oxygen species

(ROS) (Wang *et al.* 2015). As already explained, the toxicity of As^{V} is due to its structural similarity to inorganic phosphate, and the replacement of phosphate by As^{V} in glycolytic and cellular respiration pathways (Rahman and Hassler 2014). The disruption of P metabolism by incorporating As^{V} into phosphorylated compounds, which are vital to the cycling of ATP, contributes to arsenic toxicity (Levy *et al.* 2005; Wang *et al.* 2015). Toxicity increases when detoxification mechanisms are overwhelmed under severe arsenic stress, causing oxidative stress and cell division inhibition in microalgae (Levy *et al.* 2005; Wang *et al.* 2015). However, studies conducted with algal cultures demonstrated that increases in P in the culture media can significantly decrease the toxicity of As^{III} and As^{V} (Wang *et al.* 2015). For instance, increasing phosphate concentration from 1 µM to 10 µM (Guo *et al.* 2011) may decrease the growth inhibition of freshwater microalgae by arsenic and enhance the tolerance to arsenate (Levy *et al.* 2005; Guo *et al.* 2011), but not under excess phosphate in the medium (e.g. 175 µM in Guo *et al.* 2011). However, discrepancies may be found in literature analyzing arsenic uptake and toxicity as a function of P availability when comparing supposedly equal phosphate conditions (limiting or non limiting) among different studies. The reason could probably be due to the noted differences in literature concerning which P concentrations are considered as P-limited or non-P limited conditions (e.g. excess P is 20 µM for Hellweger *et al.* 2003; whereas it is 175 µM for Guo *et al.* 2011). Moreover, not only in the environment or in the culture medium, but also small variations of intracellular phosphate concentrations could significantly change the toxicity of As^{V} in microalgae (Hellweger *et al.* 2003; Wang *et al.* 2013). Therefore, in addition to the analysis of the total cellular arsenic content, a better predictability of the arsenic toxicity in microorganisms may be achieved by analyzing the cellular As/P ratio, as already done in some studies (e.g. Levy *et al.* 2005; Wang *et al.* 2013; Rodriguez-Castro *et al.* 2015), and future research should take it into account.

Some arsenic ecotoxicity data for biofilms (periphyton) and diatoms is showed on Table 2, compiled from the Pesticide Action Network (PAN) Pesticide Database (http://www.pesticideinfo.org/List_AquireAll.jsp?Rec_Id=PC35165&Taxa_Group=Phytoplankton) These data were measured using different toxicity endpoints and include IC20 values (concentrations of arsenic that induced 20% inhibition relative to controls), LOEL or LOEC values (the "lowest observed effect level," or the lowest concentration at which adverse effects are observed), and NOEL or NOEC values ("no observed effect level" or the concentration below which no adverse effects are observed). Mean arsenic toxicity values for periphyton are ranging from 15 to 59.9 µg As L^{-1}; while for diatoms values may range from 25 to 150 µg As L^{-1}. Toxicity values of arsenic species (As^{V} and As^{III}) for algae (Table 2), were compiled from the ECOTOXicology knowledgebase (ECOTOX) (http://cfpub.epa.gov/ecotox/) by Tuulaikhuu (2016), including the LC50 value (concentration of a compound causing 50% mortality of the tested organisms) set at 79.4 mg As^{V} L^{-1}, and the NOEC values set at 1.19 mg As^{V} L^{-1} and 8.59 mg As^{III} L^{-1}. Comparing both databases, the PAN shows higher variability on its values that, in turn, are always much lower concentrations than in the ECOTOX database and are also more in concordance with the real environmental arsenic ocurrence. Moreover, the ECOTOX database

is very limited in terms of groups (e.g. it includes no LC50 value of As^{III} for algae) and has low ecological realism because it is mainly based on acute toxicity to a single species. Investigating arsenic toxicity after longer exposure and/or at a larger scale of biological organization is crucial to account for the effects that exposure may cause on the structure and function of aquatic communities and ecosystems (Tuulaikhuu 2016).

In biofilms, it has been demonstrated that As^{V} exposure may lead to changes in their structure and function. It was also detected that environmental phosphate concentration may mitigate As^{V} uptake and the resulting toxicity in biofilms. For instance, chronic exposure (4 weeks) to 130 µg As^{V} L^{-1} affected structural and functional parameters of epilithic biofilm communities starved of P, inhibiting algal growth, photosynthetic capacity, changing the algal community composition, reducing its ability to retain P and accumulating more arsenic into the cells. Arsenic tolerance was only induced by the combination of As^{V} and high P treatments indicating that tolerance induction may be an ATP-dependent mechanism. In addition, it was also shown that arsenic retention was reduced under high-P conditions (Rodriguez Castro *et al.* 2015).

Table 2 Mean arsenic toxicity values for different exposed organisms (Biofilm, Algae and Diatoms). NR: not reported toxicity endpoint.

Database	As-exposed organisms	Mean toxic dose	Concentration units	Toxicity endpoint	Measurement
PAN	Biofilms (Periphyton)	37.5	µg As L^{-1}	IC20	C content
		59.9			N content
		44.9			Photosynthesis
		30			
		22.5			
		22.5			Biomass
		15			Diversity
	Diatoms	60	µg As L^{-1}	NR	General Biochemical effects
		150			
		25			
		1.5	pg $cell^{-1}$	LOEC	Abundance
		4.5		NOEC	
ECOTOX	Algae	79.4	mg As^{V} L^{-1}	LC50	-
		1.19		NOEC	-
		8.59	mg As^{III} L^{-1}	NOEC	-

3.1.1 The sensitivity of diatoms to metal toxicity, and causes and benefits of diatom size reduction

Diatoms usually represent the major autotrophic proportion of biofilms (Navarro *et al.* 2002; McClellan *et al.* 2008; Morin *et al.* 2010), being widely used as biological indicators of water quality (Kelly *et al.* 1998; Prygiel *et al.* 2002). Diatom communities are likely to accumulate significant quantities of metals (Guanzon *et al.* 1995; Sunda and Huntsman 1998; Chang and Reinfelder 2000; Wang and Dei 2001). Numerous works have described the mechanisms of toxicity of metals for diatoms; however, the toxicity caused by some metalloids, arsenic in particular, has not been such extensively studied, although similar effects may be expected. In general, metal toxicity causes effects on diatom multiplication, photosynthesis, respiration, assimilation of nutrients and synthesis of molecules (Morin 2006). Consequently, several diatom metrics have been applied in ecotoxicology to assess metal pollution, at the community level (through shifts in dominant taxa and diversity patterns) and also at the individual level, with the appearance of teratological forms (e.g. Falasco *et al.* 2009; Ferreira da Silva *et al.* 2009; Luís *et al.* 2011; Lavoie *et al.* 2012) and size decrease (e.g. Cattaneo *et al.* 1998; Ivorra *et al.* 1999; Morin *et al.* 2007). However, in spite of the well-known response of diatom communities to metals, no diatom index for metal pollution assessment has still been developed (Jüttner *et al.* 2012), and it remains also work to be done in relation to metalloid pollution assessment, as in the case of arsenic.

The production of ROS induced by metal toxicity causes damage in cells, resulting in increasing mortality and growth inhibition due to disturbances in cell division. Under these conditions, asexual reproduction is the favored mode of multiplication in diatoms, leading to a selection of the smallest individuals. Additionally, interferences with the metabolism of silica appear to be responsible for the appearance of deformed frustules. Primary production (algal growth and photosynthetic activity) is also especially affected by exposure to these toxic elements (Morin 2006).

Metals also cause changes in the structure and architecture of diatom communities in biofilms, in relation with the different diatom-specific levels of tolerance to metals (Paulsson *et al.* 2000; Gold *et al.* 2002). In fact, small and early successional species (e.g. *Achnanthidium minutissimum*, Fig. 7; and *Fragilaria vaucheriae*) are usually more tolerant to metals than the late successional communities, dominated by colonial and filamentous species (e.g. *Melosira varians* and *Diatoma vulgare*) highly sensitive to metals (Medley and Clements 1998). The sessile mode of living and their small size enable this species to occupy different spatial positions in the community, suggesting a certain tolerance to different nutrient and light conditions, and metal exposure (Ivorra *et al.* 1999). Therefore, it is common to observe a decrease in the specific richness and diversity of the communities exposed to metals in their initial stage of development (Medley and Clements 1998; Niyogi *et al.* 2002; Gold *et al.* 2003).

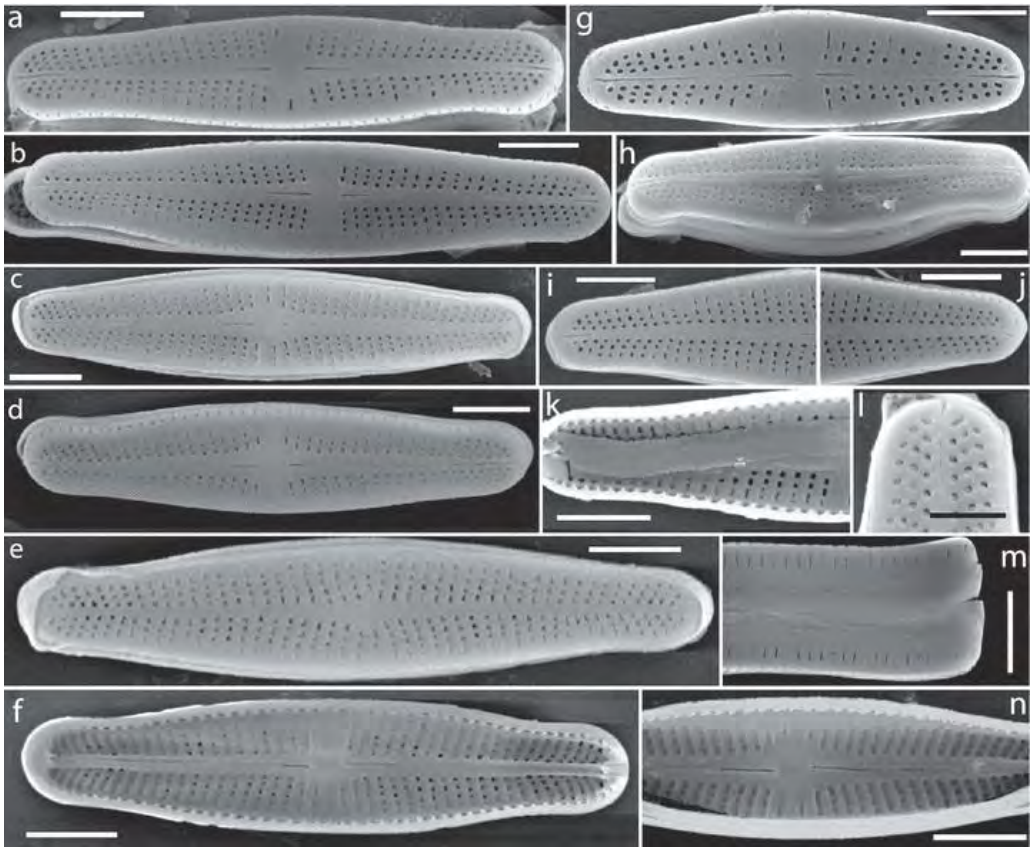


Figure 7 Scanning electron micrographs of the type material of *Achnanthidium minutissimum*. (a–d, g, h) External views of whole raphe valves. (e) External view of a rapheless valve. (f) Internal view of a raphe valve. (i, j, l) External views of valve fragments; (i) and (j) are fragments of the same valve showing straight (i) and slightly deflected (j) terminal raphe ends. (k) Internal view of a valve fragment with a copula. (m) Girdle view showing valve mantle and copulae. (n) Internal view of the central part of a valve showing proximal raphe ends slightly deflected in opposite directions. Scale bars, 2 μm. From Patapova and Hamilton 2007.

Overall, metal exposure is linked to a reduction in the thickness of the biofilm, compared to biofilms developed under non-metal-stress conditions (see Fig. 8), indicating the importance of cell size reduction as a response of toxicity in diatoms.

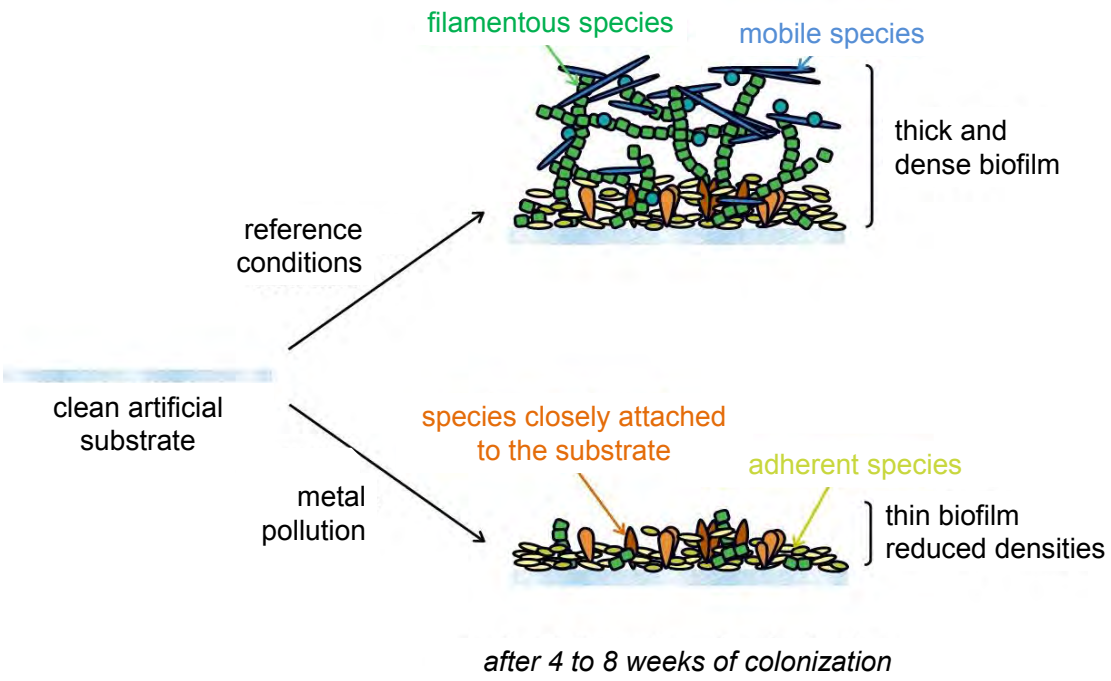


Figure 8 Schematic representation of the effects of metal contamination on the architecture of diatomic communities developing on clean artificial substrates under controlled experimental conditions (modified from Morin 2006, after Gold 2002).

✓ **The influence of other environmental factors on the diatom size reduction**

In addition to metal or metalloid exposure, diatom size decrease may be induced by the effects of other environmental factors, such as nutrient limitation/depletion, light intensity and environmental temperature. For instance, decrease on diatom cell size was reported for the freshwater *Cyclotella meneghiniana* and the marine *Chaetoceros muelleri*, *Thalassiosira weissflogii*, *Phaeodactylum tricornutum* under Fe depletion (Geider *et al.* 1993; Davey and Geider 2001; Beer *et al.* 2011). Iron is an important factor in photosynthesis, respiration, and nitrogen fixation (Morel and Price 2003) and, thus, one of the trace metals that are essential for the growth of microalgae (Beer *et al.* 2011). Other example of nutrient limitation effects was suggested in Rodriguez-Castro *et al.* (2015), where a decrease of P uptake under chronic arsenic exposure would cause diatom cell-size decrease, although no significant relationship between diatom size and TP has been detected before (e.g. Lavoie *et al.* 2006).

Light intensity during growth is a decisive factor for all photosynthetic organisms and leads to adaptations of the photosynthetic apparatus and the overall cell structure (Beer *et al.* 2011), such as decrease of cell and chloroplast size in different diatom species when are exposed to high light conditions (140 µmol photons m^{-2} s^{-1}) (Rosen and Lowe 1984; Kudo *et al.* 2000; Davey and Geider 2001; Janssen *et al.* 2001). These changes are reflected by alterations at the molecular level, such as on the function of the PSII (Beer *et al.* 2011).

Regarding temperature, diatoms appear to dominate in temperate to cooler regimes, and an inverse relationship between organism size and rearing temperature in ectotherms has

been widely observed. For each 1 °C increase, a cell-size reduction of 2.5% (95% CI of 1.7-3.3%) of the volume was observed at 15 °C in Atkinson *et al.* (2003), and the value did not differ across taxa (amoebae, ciliates, diatoms, dinoflagellates, flagellates), habitats, modes of nutrition or combinations of these. Most of the studies that have analyzed cell size of freshwater and marine photosynthetic microorganisms, including diatoms, are aiming to evaluate effects of the global change. Additionally, possible evolutionary causes for cell size decrease include adaptation to changes associated with increasing temperature such as, on one hand, decrease of O_2, CO_2 or some nutrient concentrations and, on second hand, faster generation times (Atkinson *et al.* 2003; Finkel *et al.* 2010). All these effects caused by changing temperature could likely be taken into account for ecotoxicological studies. However, the link between microalgal cell size and the effects of warming or other factors (e.g. nutrient addition or presence/absence of fish) is sometimes uncertain (e.g. Moss *et al.* 2003).

3.2 Arsenic toxicity to fish

Several studies have detected biochemical changes and genotoxicity effects on fish due to arsenic exposure (e.g. Castro *et al.* 2009; Ventura-Lima *et al.* 2009; Kumar *et al.* 2014; Tuulaikhuu *et al.* 2016), with concentrations ranging from 10 to 100 µg As L^{-1}. Under lower aquatic concentrations (around 2 µg As L^{-1} in water) but with higher values in sediments (ranging from 10 to 14 mg kg^{-1} or ppm), consistent negative relationships between fish size and environmental arsenic concentrations was detected in different fish species (Merciai *et al.* 2014). Regarding arsenic species, values for fish from the ECOTOX database are set at higher concentrations (Tuulaikhuu 2016), establishing the LC50 values at 40.9 mg As^{V} L^{-1} and 24.5 mg As^{III} L^{-1}.

Arsenic toxicity to fish may be studied using a wide variety of biomarkers ranging from, for instance, molecular analyses such as enzyme activity determination (e.g. Tuulaikhuu *et al.* 2016) to analyses related to fish physiology and behavior. The effects of arsenic toxicity have been examined in numerous species worldwide. However, most research has focused on parameters such as bioaccumulation, and physiological parameters such as growth (e.g. Kumar and Banerjee 2012) and metabolic and histopathological effects (e.g. Ahmed *et al.* 2013; Bhattacharya *et al.* 2007). One factor that has received much less attention is fish behavior (e.g. Scott and Sloman 2004; Weis and Candelmo 2012; Weis *et al.* 2001). Contamination in natural systems is often at concentrations well below those that cause mortality, but even low levels of toxicity may be sufficient to interfere with normal functioning. Fish behavior is ideal for assessing these sublethal impacts (Moss 1998; Scott and Sloman 2004; Weis and Candelmo 2012). Alteration of complex, naturally occurring behaviors such as foraging and predation, agonistic interactions, shoaling and reproductive behaviors are more ecologically relevant indicators of toxicity (Scott and Sloman 2004; Sopinka *et al.* 2010; Weis *et al.* 2001).

3.3 Influence of biofilm-fish interaction on the arsenic toxicity

Biofilms constitute important sources of energy for invertebrates and herbivorous fish (Stevenson *et al.* 1996). Moreover, they are not only a site for biotransformation but also a site of transfer of chemicals to higher organisms (Guasch *et al.* 2016). Direct effects of toxicants on the most sensitive community (e.g. microalgae and/or prokaryotes) may lead to indirect effects on the rest of biofilm components (e.g. Proia *et al.* 2012), and also on higher organisms of the food web, since all of them are closely related through biological interactions (Guasch *et al.* 2016). Under natural conditions, the interaction between biofilm and fish is also related to the nutrient cycling, which is a crucial process in the ecosystem functioning. Actually, fish play an important role as nutrient subsidies, while biofilm actively uptakes the nutrients, playing a role in water purification and increasing productivity in the subsidized system. However, arsenic may change nutrient dynamics and, finally, influence the whole ecosystem (Tuulaikhuu 2016). For instance, Tuulaikhuu *et al.* (2015) assessed the effects of 120 μg As^{V} L^{-1} to periphyton, epipsammon and fish under P-limiting conditions (around 6 μg L^{-1}). Total dissolved arsenic concentration decreased exponentially to 28 μg As L^{-1} during the experiment (60 days), mostly sinking to the sediment, and a small percentage accumulated in the periphyton. Most P and N were also retained in the epipsammon. Arsenic effects to fish were decreased in the presence of biofilms at the beginning of exposure (day 9) but not later on, since the arsenic-affected biofilm had lost its role in arsenic detoxification. After longer exposure (56 days), arsenic reduced the total biomass of biofilm and its potential ability to use organic P (i.e., phosphatase activity), inhibiting algal growth, especially that of diatoms, and making the epipsammon more heterotrophic. In conclusion, biofilms are the basis for primary production in rivers, consequently their quality and quantity may influence the ecosystem's health and fitness of higher organisms such as fish. Moreover, communities of microorganisms in biofilms contribute also to nutrient cycling, and arsenic may influence this ecosystem service.

4. EXAMPLES OF ARSENIC-IMPACTED SITES

In this section, two particular As-impacted rivers are shown, as examples of natural and anthropic (gold-mining activities) arsenic pollution, being the cases which this thesis was inspired by. The different dynamics of the arsenic cycle depending on the source of contamination (sediment *vs.* water) are also exposed.

4.1 Pampean Streams: Effects of Naturally Occurring Arsenate in Surface Waters

The Chaco-Pampean plain is a vast area of Argentina (over 1×10^{6} km^{2}) and one of the greatest obstacles for the socioeconomic development of the region is the availability and quality of the groundwater for the peri-urban and rural population (Viglizzo and Jobbágy 2006). Numerous articles have addressed the quality of Pampean groundwater since the detection of arsenic (Nicolli *et al.* 1989; Smedley *et al.* 2002; Farias *et al.* 2003; Nicolli *et al.* 2010) but just recently, the presence of arsenic in surface waters (Galindo *et al.* 2007; Schenone *et al.* 2007;

Puntoriero *et al.* 2014) and particularly in lotic environments (Rosso *et al.* 2011; Rodríguez Castro 2015) has been noticed.

In recent studies in the province of Buenos Aires, part of the Pampean region, Rosso *et al.* (2011) have surveyed arsenic levels in 39 Pampean streams, finding values higher than the recommended for the protection of the aquatic biota (average 114 μg L^{-1}), which is unusual in undisturbed natural systems. Furthermore, these streams have exhibited a wide range of PO_4^{3-} concentrations (Feijoó and Lombardo 2007). Arsenic levels in surface waters were correlated with water conductivity (Rodríguez Castro 2015) and the most contaminated fluvial systems (Freguelli 1956) are located in the south of Buenos Aires, corresponding to Vallimanca stream tributaries and Atlantic Ocean tributaries. The highest level has been found in Chasicó stream, with 198 μg L^{-1} (Rosso *et al.* 2011).

Arsenic levels in surface waters have been attributed to the hydrogeology of the streams, fed by an aquifer with high concentrations of arsenic (0.6 to 4.9 mg L^{-1}). Several of these studies have suggested that the source of arsenic in the Argentine groundwater is the Cenozoic loess sediments present in most of the aquifers (Smedley *et al.* 2005) and volcanic glass ashes in these sediments (López *et al.* 2012; Mukherjee *et al.* 2013) that is mobilized in aerobic or oxidized and high pH hydrogeochemical conditions. Studies on hydrological and chemical conditions under which the interface between the stream and catchment interface (SCI) influences the release of reactive solutes into stream water during storms have suggested that the provenance of arsenic in surface water of Pampean streams is groundwater (Rodríguez Castro *et al.* 2016). Arsenic bioaccumulation has been studied in fish, filamentous algae and biofilm (Schenone *et al.* 2007, 2014; Rosso *et al.* 2013; Rodríguez Castro 2015) but no studies on biomagnification have been performed. Arsenic levels in fish tissue are among the highest reported worldwide (Petkovšek *et al.* 2012; Noël *et al.* 2013), ranging from 1.04 to 3.547 μg gDW^{-1}. Large differences between fish species have been observed (Rosso *et al.* 2013). Other studies have shown that arsenic concentration ranges from 2.9 to 33.9 μg gDW^{-1} in filamentous algae and from 14.2 to 214.7 μg gDW^{-1} in biofilms, indicating that no biomagnification occurs with this metalloid (Rodríguez Castro 2015). Also, no correlation has been found between arsenic levels in the water column and those accumulated by these organisms, suggesting that its bioavailability depends on factors other than dissolved arsenic concentration. Dissolved phosphorus, humic acids and sediments are elements that may influence arsenic bioavailability (Buschmann *et al.* 2006; Sharma and Kappler 2011; Chakraborty *et al.* 2012).

At the community scale, field colonization allowed natural exposition of biofilm communities to arsenic and P. Periphytic communities developed in 7 Pampean streams with different arsenic and P concentrations have revealed differences in structural and functional parameters. The minimum fluorescence (*Fo*), structural parameter that responds to long term disturbances, has responded with a linear trend to arsenic and P (*Fo*= 216.4 + 0.947SRP – 2.11As. Linear Regression, $p<0.09$). Communities grown in streams with high arsenic levels have shown low *Fo*, suggesting that chronic exposure to arsenic has a negative effect on

periphytic growth and that this effect was palliated by the presence of high levels of P (Rodríguez Castro 2015).

Therefore, high naturally occurring arsenic concentrations are found in the surface waters of the Pampean streams and this is considered an important health issue, but little is known about its environmental impact. More research is needed to better understand the bioavailability and biomagnification of arsenic in the organims living in these peculiar streams.

4.2 The Anllóns River: polluted sediments resulting from former mining activities

In the Anllóns River (Galicia, NW Spain; see Fig. 1 of the ***Chapter 3***), high arsenic concentrations are found in surface and subsurface sediments which are attributed to natural geogenic arsenic enrichment exacerbated by mining activities (Devesa-Rey *et al.* 2008). Arsenopyrite mineralization in hydrothermal quartz veins (Nespereira 1978) is associated to gold ores which were exploited during the Roman Empire and then from 1895 until 1910, with intermittent withdrawals after that period. Arsenic concentrations in the rocks of the area are usually around 1%, but in mineralized zones with semi-massive arsenopyrite they can reach up to 10 %. In the superficial soil horizons in the mineralized areas, arsenic contents of 4,000 mg kg^{-1} have been detected (Boixet *et al.* 2007). In the riverbed sediments high concentrations that can reach 264 mg As kg^{-1} were detected downstream the mineralized area to the river mouth (Devesa-Rey *et al.* 2008; Rubinos *et al.* 2010). Costas *et al.* (2011) found even higher values (up to 308 mg kg^{-1}) at the estuary and estimated that the Anllóns River exports to its estuary 460 kg y^{-1} of dissolved (< 7% as organic) arsenic annually.

Geochemical investigations using sequential extractions showed that most arsenic in the sediments of the Anllóns River is associated to low-mobility phases (Devesa-Rey *et al.* 2008; Rubinos *et al.* 2011b), specifically as bound to Fe-oxides forms and in the residual phase. This low arsenic solubility was confirmed by the results of availability tests, addressed to estimate the leaching potential of arsenic and its effect on the survival of microorganisms (TCLP extraction), the bioavailability to higher plants (1 M HCl) and the bioavailability to superior animals (PBET). This latter extractant solubilized the highest arsenic concentrations, not exceeding 11% of the total arsenic (Devesa-Rey *et al.* 2008).

Nevertheless, it has been demonstrated that this apparent low arsenic mobility may vary with changes in the environmental conditions, which poses a potential environmental risk. Arsenic release from the contaminated sediments increases with increasing water:sediment ratios, suggesting an increased risk of mobilization during high-flow resuspension events (Rubinos *et al.* 2010). Also, as mentioned above, arsenic mobility is strongly dependent on the pH, and it occurs simultaneously with the dissolution of components with which it is associated: oxides and hydroxides of Fe and Al at acidic pH, and organic matter at alkaline pH (Rubinos *et al.* 2011b). Arsenic release is also promoted in high ionic strength conditions, as it is characteristic of estuarine environments where the mixture of fresh and marine waters occurs (Rubinos *et al.* 2011b). It is favored by the presence of phosphate, showing high concentrations in some sections of the river (Iglesias *et al.* 2011; Barral *et al.* 2012; Rial 2007). It comes from

wastewaters discharged into the river course and fertilizers eroded or leached from the soils of the basin. Interestingly, although phosphate favors arsenic release from the Anllóns sediments, it was shown in Microtox® bioassays that it counteracts the acute toxicity of As^{V}, but has no effect on the toxicity of As^{III} and DMA^{V} (Rubinos *et al.* 2014).

The biological status of the river sediments also affects arsenic biogeochemistry. The bed sediments of the Anllóns River are covered by epipsammic biofilms (Devesa-Rey *et al.* 2009), mainly constituted by *Bacillariophyceae* (diatoms) which represent >86 % of the total abundances in the superficial sediments (Martiñá Prieto *et al.* 2016). Epipsammic biofilm inocula from this river have been satisfactorily incubated at a laboratory scale in experimental fluvial channels and bioreactors (Prieto *et al.* 2016b), having effects on the transfer and speciation of As^{V} in the water column and sediment. Thus, batch adsorption experiments performed with sediments covered by epipsammon revealed that biofilms increased the retention of dissolved arsenic by the Anllóns sediments (Prieto *et al.* 2013), particularly in the presence of phosphate, which had a negative effect on arsenic retention in the systems devoid of biofilm (Fig. 9). This behaviour was confirmed in bioreactor experiments conducted with unpolluted sediments from this river exposed to As-enriched river water, which showed that the biofilms increased the retention of As^{V} (up to ~97 %) from the water column in comparison with the sediment without biofilm (~70 %) (Prieto *et al.* 2016c). The biofilm also avoided the reduction of As^{V} to As^{III} in the water column and promoted the occurrence of organic species such as MMA^{V} and DMA^{V} which result from biological transformations (Prieto el al. 2016b). Most of the arsenic in the biofilm was retained in extracellular compartment (~71 %), almost exclusively in the form of As^{V} (~99.5 %).

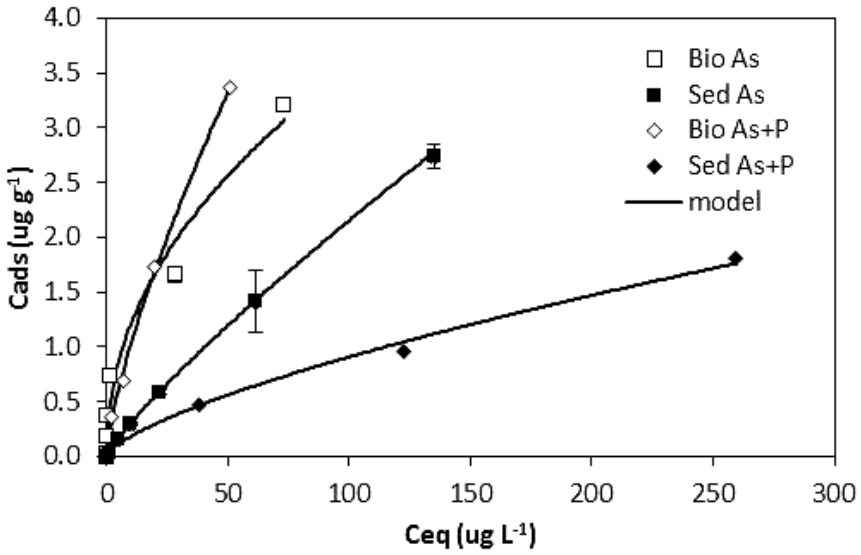


Figure 9 Effect of biofilm and phosphate addition on arsenate retention by sediments. Retention experiments were carried out at pH 5.5 and arsenic concentrations assayed were 0, 5, 25, 50, 100, 250 and 500 µg L^{-1} prepared in Milli-Q water and 0.01 M $CaCl_2$ as background electrolyte. C_{eq}: Equilibrium arsenic concentration in solution. C_{ads}: Adsorbed arsenic concentration by sediments (Sed arsenic and Sed As+P indicate that sediments are exposed to arsenic concentrations and to arsenic and P concentrations at equimolar ratios, respectively) and by sediments covered by biofilm (Bio arsenic and Bio As+P). Figure modified from Prieto *et al.* (2013).

In turn, when studying arsenic mobility from As-polluted Anllóns sediments, Prieto *et al.* (2016c) showed that the biofilm reduced by 64 % the release of total arsenic to the water column in comparison with sediments without biofilm, and avoided the reduction of As^{V} to As^{III}. This fact has toxicological relevance due to the usually higher mobility and toxicity of As^{III} (Oremland and Stolz 2003; Sharma and Sohn 2009). In this case, the arsenic retained by the biofilm was equally distributed among the intracellular and extracellular compartment. Inside the cells, significant concentrations of As^{III}, MMA^{V} and DMA^{V} were detected, suggesting that active methylation and detoxification processes occurred in the intracellular compartment. Both in retention and mobilization studies by biofilms, volatilization did not play a key role in the global cycle of arsenic.

Overall, these studies performed in the Anllóns River have put forward the relevance of epipsammic biofilms on the behavior of arsenic in freshwater environments, by promoting arsenic retention, inhibiting reduction of As^{V} to As^{III} as a final product, performing biomethylation, and thus reducing its potential toxicity to the environment.

5. WHAT DO WE STILL HAVE TO UNDERSTAND AND WHY?

With this thesis, we try to provide more information to the knowledge of arsenic toxicity and biogeochemistry in rivers, with special focus on periphyton or benthic biofilm since they can be affected by the toxicity and, moreover, change the biogeochemistry. We did different experiments to check different aspects of the environmental arsenic impact. First, we wanted to understand the toxicity of arsenic to “epilithic” biofilms and, particularly to microalgae. To do that, a laboratory experiment was performed. Despite the highly important information that diatoms can give about the ecological state of the rivers, information about arsenic effects on freshwater diatoms (and microalgae in general) is really lacking in the literature. Effects on epipsammic biofilm (including diatoms) have been study before (e.g. Martiñá Prieto *et al.* 2016) but not in epilithic biofilms. Then, we analyzed the interaction between biofilm and fish during arsenic exposure with the aim of improving the ecological realism in our experiments. Very few ecotoxicologists include different trophic levels in their studies despite the important information that they can give to understand the direct and the indirect effects of a toxic exposure. Interactions biofilm-fish in arsenic experiments, as the one done by Tuulaikhuu 2016, are not in abundance in the literature; however, in that study, structural effects on biofilms and particularly on diatoms were not addressed. In this thesis, these effects are presented. Finally, and once we observed and understood the responses of biofilm to arsenic exposure under control conditions at a mesocosm level, we could move forward to a more complex and real scenario doing a field experiment.

6. GENERAL OBJECTIVES AND HYPOTHESES

Based on the current knowledge about biofilms ecotoxicology and arsenic biogeochemistry in freshwater ecosystems, this thesis aims to study, under realistic

environmental arsenic concentrations, **i)** the toxic effects of arsenic on the structure and function of benthic fluvial biofilms, with especial attention to diatom responses, **ii)** the interaction among these As-exposed primary producers and As-exposed higher organisms (fish), and **iii)** the role of benthic biofilms on As-bioavailability and As-detoxification in a freshwater system

To achieve these main objectives, laboratory and field experiments were conducted, and several endpoints related to photosynthetic activity and algal biomass, bacteria density and diatom taxonomy changes were carried on. Laboratory experiments were performed using natural arsenic concentrations, as those of the Pampean streams (130 µg As L^{-1}), to analyze the effects of aquatic arsenic in artificial fluvial systems but under natural concentrations. The effect of anthropic As-impact in fluvial systems were studied in a field experiment at the Anllóns River, where arsenic is mainly located in the sediments.

These investigations are explained in 3 chapters in this PhD dissertation, with the following specific objectives:

- ✓ ***Chapter 1*** (laboratory experiment): To investigate the effects of short-term arsenate exposure on the structure and function of fluvial biofilms, and especially on the diatom community, under the influence of fish (*Gambusia holbrooki*) on nutrient cycling.
- ✓ ***Chapter 2*** (laboratory experiment): To analyze whether biofilm may reduce arsenic toxicity on fish (*Gambusia holbrooki*), through a possible P supply in water coming from fish excretion, leading to a decrease on As^{V} uptake into biofilm cells and, consequently, reducing or avoiding the excretion of higher toxic As-species (e.g. As^{III}) into the water.
- ✓ ***Chapter 3*** (field experiment): To assess the influence of benthic biofilms on arsenic retention, transformation and mobilization in a real and eutrophic freshwater system (Anllóns River), where high arsenic concentrations in soils and river bed sediments were found due to past mining activities.

The following **hypotheses** have been formulated:

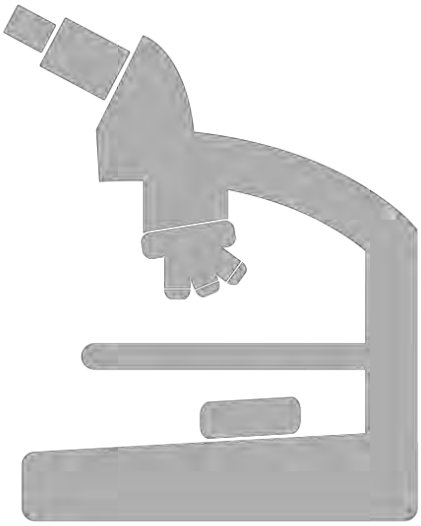
i) In a multi-trophic and ecologically realistic scenario, we expect to see interactions between different trophic levels as biofilm and fish: we hypothesize that arsenic toxicity to biofilms will modify arsenic toxicity to higher organisms (fish); and that fish metabolism will influence trophic conditions in the system, increasing phosphate concentration and, therefore, alleviating arsenic toxicity in microalgae.

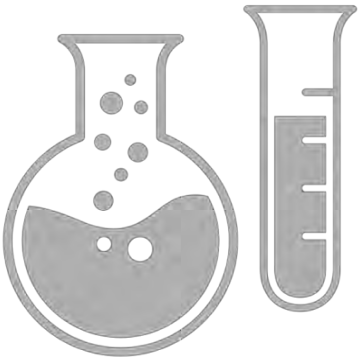
ii) We predict that arsenic toxicity on biofilms will affect the structure and function of microalgae.

iii) We assume that phosphate conditions modulate arsenic toxicity for primary producers, hoping to identify different arsenic effects on biofilms depending on the different phosphate concentrations (mainly, detecting toxicity decrease when phosphate increases).

iv) We expect to detect the contribution of fluvial biofilms to the mobilization and speciation of arsenic in freshwater systems, modulating arsenic toxicity in the environment (probably, through detoxification processes, such as methylation).

2. MATERIALS AND METHODS





In this general section of Materials and Methods, the experimental designs and the main techniques used in the thesis are indicated. Different analyses were done in the three studies of this thesis. Laboratory experiments **(*Chapter 1*** and ***2*)** were performed using 12 artificial stream channels, while the field experiment **(*Chapter 3*)** was conducted in an Atlantic river: the Anllóns River (Galicia, NW Spain). In all these studies, biofilms were developed on artificial hard substrates (glass tiles), which are typically used in biofilm investigations as substitutes for natural rocky substrates (Mora-Gómez *et al.* 2016). The methodology followed in this thesis is summarized in this section but described in more detail within each chapter.

1. EXPERIMENTAL DESIGNS

Laboratory experiments **(*Chapter 1*** and ***2*)**

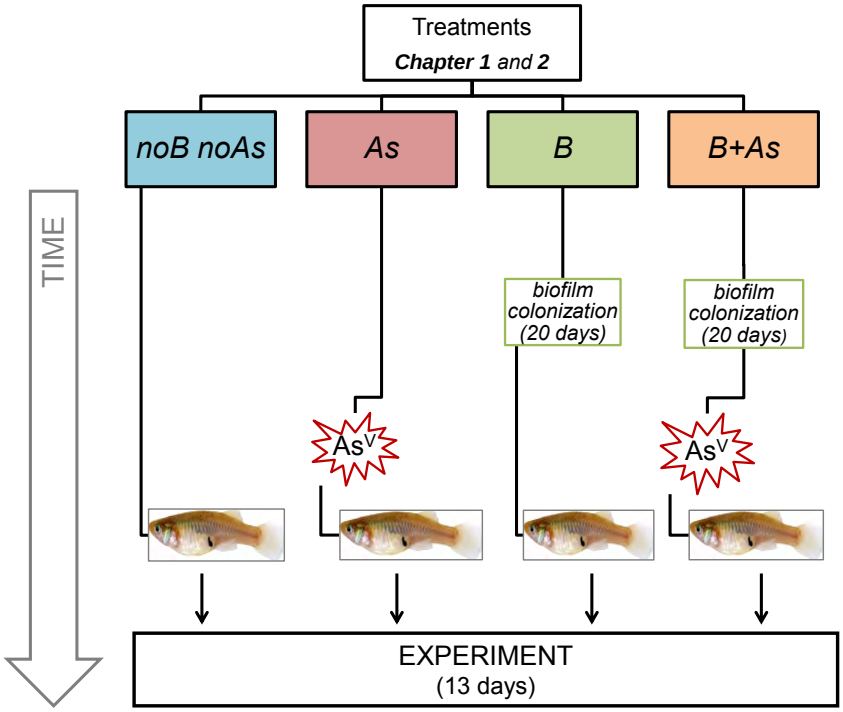
For the laboratory experiments, experimental units consisting in artificial channels simulating streams were used, some of them with colonized natural-biofilm on the bottom, and all of them with fish (placed separately). Different treatments were constituted with biofilms, fish and arsenic (Fig. 1.a):

- ✓ *noB noAs* (without biofilm or arsenic) in ***Chapter 1***, named "*control*" in ***Chapter 2***
- ✓ *As* (with arsenic only) in ***Chapter 1***, named *A* in ***Chapter 2***
- ✓ *B* (with biofilm only) in ***Chapter 1*** and ***2***
- ✓ *B+As* (with both biofilm and arsenic) in ***Chapter 1***, named *B+A* in ***Chapter 2***.

Field experiment **(*Chapter 3*)**

For the field experiment, the experimental units consisted on cement cobbles with fixed glass tiles colonized by natural biofilm, placed horizontally upstream and downstream a mine area in the Anllóns River riverbed and, therefore, exposed to different arsenic concentrations. The two sites constituted the different treatments of this study (Fig. 1.b).

a)



b)

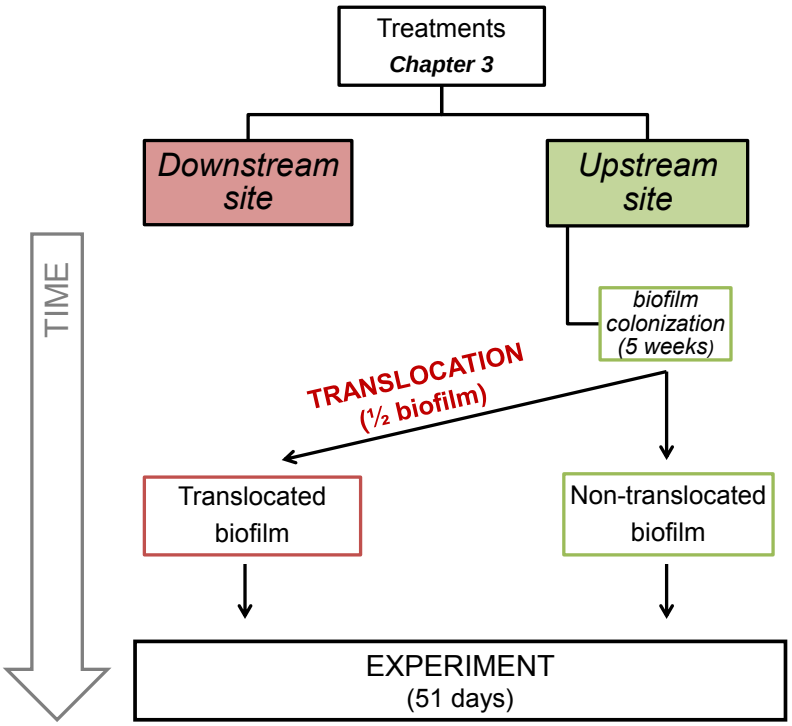


Figure 1 Diagrams of the experimental designs peformed in the different studies of this thesis. Specifically, it is shown how it was performed or considered the different treatments in a) ***Chapter 1*** and ***2***; and b) in ***Chapter 3***. See main text on the respective chapters for more information.

2. MAIN ANALYTICAL METHODS

Environmental light intensity (µmol photons m^{-2} s^{-1}), using a light sensor (LI-COR Inc., Lincoln, Nebraska, USA), in ***Chapter 3***:

- ✓ *Riparian cover*
- ✓ *Light reaching benthic biofilm*

Water chemical analyses

Chapter 1 and ***2***:

- ✓ *Physical* and *chemical parameters* (water temperature, dissolved oxygen, electrical conductivity and pH) using HQ Portable Meters, HQ40d18, HACH Company.
- ✓ *Inorganic phosphate (iP)* concentration, by a modified molybdenum blue method (Carvalho *et al.* 1998) to avoid arsenate interference.
- ✓ *Total dissolved arsenic concentration*, using Inductively Coupled Plasma Spectrometry (ICP-MS 7500c Agilent Technologies, Inc. Wilmington, Denmark)

Chapter 3:

- ✓ *Suspended solids* (SS; according to APHA, 1995)
- ✓ *Total dissolved nitrogen* (TN), using the Kjeldhal method (following UNE-EN 25663:1994)
- ✓ *Total dissolved phosphorus* (TP; following APHA, 2005)
- ✓ *Soluble reactive phosphorus* (SRP; according to Murphy and Riley 1962)
- ✓ *Dissolved organic carbon* (DOC), using a Total Organic Carbon Analyser Model TOC-5000 (Shimadzu, Kyoto, Japan
- ✓ *Total dissolved As*, using ICP-MS (Varian 820MS)
- ✓ *Arsenic speciation*, using High-Performance Liquid Chromatography coupled with Inductively Coupled Plasma Spectrometry, HPLC-ICP-MS (Varian Prostar 230 HPLC-Varian 820MS).

Sediment analyses (*Chapter 3)*:

- ✓ *pH* and *Eh* determination (in the field), using a HANNA HI 9025 portable pH-Eh meter equipped with a Pt combination redox electrode (Hanna Instruments, Eibar, Spain)
- ✓ *Bioavailable arsenic* measurement *in situ* using diffusive gradients in thin films (DGT) (DGT Research Ltd., Lancaster, UK).

In the <2mm fraction:

- ✓ Determination of the *particle size distribution* (2, 1, 0.5, 0.25, 0.1 and 0.05 mm) by dry sieving.
- ✓ Extraction of the arsenic content from this sediment fraction (extracted with phosphate buffer, following Gleyzes *et al.* 2002), named *easily-extractable arsenic concentrations* in the text, and further measure of total arsenic concentration by ICP-MS.
- ✓ Determination of arsenic *speciation* in the previous extracts, measured by HPLC-ICP-MS (Varian Prostar 230 HPLC-Varian 820MS).

In the <2mm fraction, after milled and sieved (<50 μm):

- ✓ *Total phosphate* (TP; following Murphy and Riley 1962),
- ✓ *Total Kjeldhal nitrogen* (TN, following Guitián and Carballas 1976)
- ✓ *Total organic matter* (OM), through calcinations at 450 °C during 2h following the UNE-EN 13039 standard (AENOR 2012).
- ✓ Determination of *total arsenic concentration*, (following Devesa-Rey *et al.* 2008), using a X-ray fluorescence spectrometry (custom built, equipped with a Philips high-voltage generator and a Mo anode of 2.2 Kw as X-ray source).

Biofilm measurements

Chapter 1:

- ✓ *Chlorophyll-a fluorescence-related endpoints,* using PhytoPAM (Pulse Amplitude Modulated) fluorimeter (HEINZ WALZ, Effeltrich, Germany)
- ✓ *Benthic chlorophyll-a,* extracted with 90% acetone (following the method described in Jeffrey and Humphrey 1975)
- ✓ *Bacterial abundance (life-dead method)*, using the double staining Live/Dead BacLight Bacterial Viability Kit (Molecular Probes), and subsequent cells counting using epifluorescence microscopy at a magnification of 1000x in immersion oil (Nikon E600, Tokyo, Japan).
- ✓ *Diatom community identification* (following Leira and Sabater, 2005, for samples preparation; Krammer and Lange-Bertalot 1986–1991 for species identification), and *diversity indices* (Shannon and Weaver 1949; Pielou 1975) using a light microscope (Nikon E600, Tokyo, Japan) with Nomarski differential interference contrast optics at a magnification of 1000x for species identification.
- ✓ *Diatom biovolume or cell size determination* (following a set of geometrical shapes proposed by Hillebrand *et al.* 1999; see Fig. 2), using a light microscope with Nomarski differential interference contrast optics at a magnification of 1000x.

Arsenic content in biofilm (using ICP-MS):

- ✓ *Total arsenic accumulated in biofilm,* previously freeze-dried and digested with HNO_3 (65%) using a high performance microwave digestion unit (Milestone, Ethos Sel, Sorisole (BG), Italy)

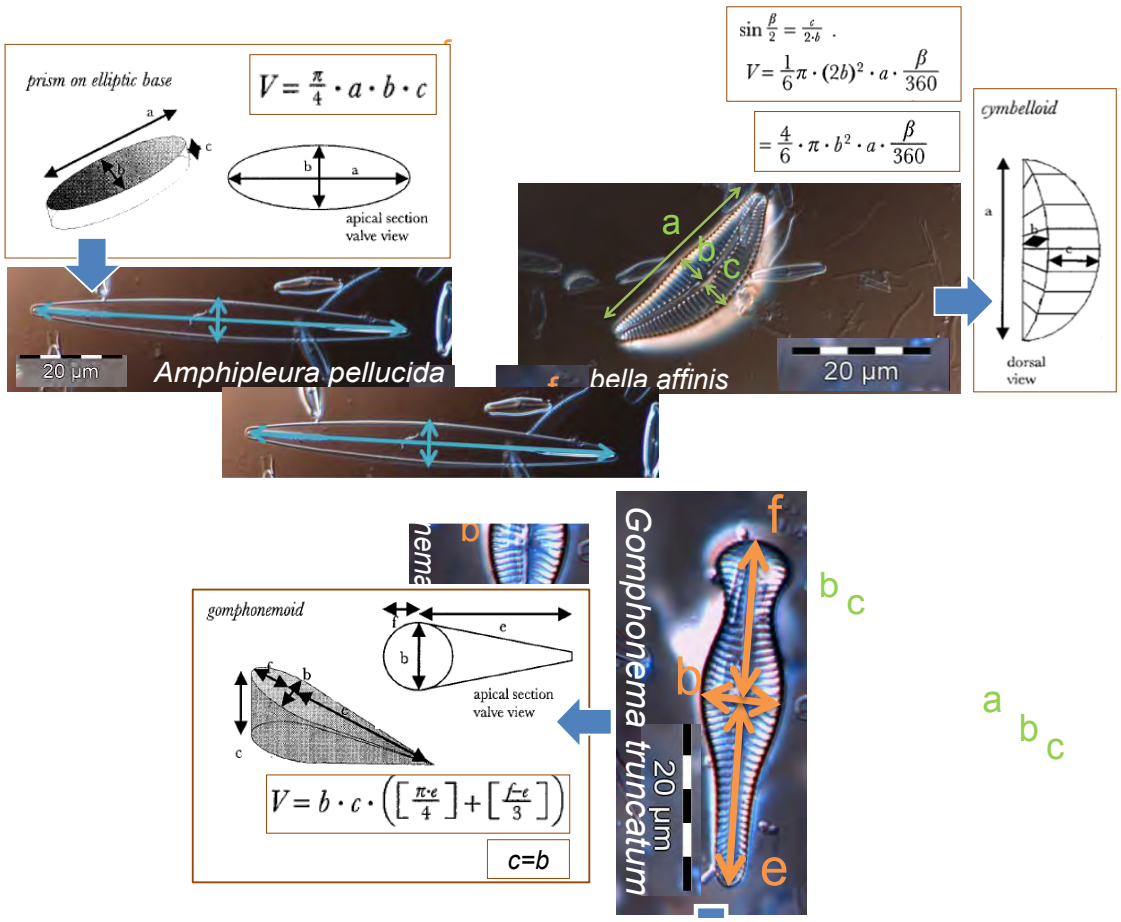


Figure 2 Examples of real measures done to diatom cells, following the set of geometrical shapes proposed by Hillebrand *et al.* (1999).

Chapter 3:

- ✓ *In vivo fluorescence measurements* (*Fo, Ymax, Yeff* parameters), using MINI-PAM fluorimeter**.**
- ✓ *Total dry weigh biomass* (DW).
- ✓ *Elemental composition* (C:N:P), using an elemental analyser (PerkinElmer 2400) for C and N; and ICP-MS (7500c Agilent Technologies, Inc. Wilmington, DE) for P determination (Sterner and Elser 2002; Muñoz *et al.* 2009; Scharler *et al.* 2015).
- ✓ *Bacterial density* (adapted from Amalfitano *et al.* 2009 and Perujo *et al.* 2015), determined by flow cytometry (FACSCalibur, Becton–Dickinson)**.**
- ✓ *Quantitative estimates of live diatom community* (following Morin *et al.* 2010), using a Nageotte counting chamber and a light microscope (Nikon E600, Tokyo, Japan).
- ✓ *Relative abundances of the diatom species* (Krammer and Lange-Bertalot 1986-1991; and Coste and Rosebery 2011) using a light microscope (Nikon E600, Tokyo, Japan) with Nomarski differential interference contrast optics at a magnification of 1000x, and *diatom diversity indices* (Shannon and Weaver 1949; Pielou 1975).

Arsenic content in biofilm*:*

- ✓ *Total arsenic accumulated* (measured by ICP-MS) in biofilm samples previously freeze-dried and digested with HNO_3 (65%) and H_2O_2 (31%) in a high performance microwave digestion unit.
- ✓ *Extracellular and intracellular arsenic content* (following Levy *et al.* 2005 for the measures in the extracellular compartment, and Myashita *et al.* 2009 for the intracellular compartment): determination of total arsenic (ICP-MS) and arsenic speciation (HPLC-ICP-MS).

Fish measurements (***Chapter 2***):

- ✓ *Direct behavior:* frequencies of operculum movements were recorded during 1 minute.
- ✓ *Complex behaviors:* the frequencies of aggressive interactions initiated for each fish (mostly females) as lunges, chases and bites were also recorded.
- ✓ *Physiological parameters:*
  - Change in biomass, by weighting fish at the beginning and at the end of the experiment.
- ✓ *Total arsenic accumulation in female fish tissue (liver and gills)* in previously frozen, freeze dried and finally digested samples with HNO_3 (65%) and H_2O_2 (31%).

Table 1 Summary of the different analytical methods used in a) environmental samples (light, river water and sediments) and b) biological samples (biofilm and fish), in each chapter (***Ch***) of this thesis.

a)

		Ch 1	***Ch 2***	***Ch 3***
LIGHT	Riparian cover			
	Light arriving benthic biofilms			
WATER	Physical and chemical parameters			
	SS			
	TN			
	TP			
	iP modified molybdenum blue method			
	SRP			
	DOC			
	Total As			
	As speciation			
SEDIMENT	pH and Eh			
<2mm fraction	Particle size distribution			
	Easily-extractable As concentrations			
	Extracts As speciation			
<2mm fraction, after milled and sieved (<50 µm)	TP			
	TN			
	OM			
	Total As concentration			
DGTs	Total As concentration			

b)

		Ch 1	*Ch 2*	*Ch 3*
BIOFILMS				
	In vivo fluorescence measurements			
	Benthic chl-a			
	DW			
	C:N:P			
	Bacterial density			
	Bacterial viability (L/D)			
	Live diatom			
	Diatom specific relative abundances			
	Diatom biovolume or cell size determination			
	Diatom specific diversity indices			
Arsenic content	Total bioaccumulated-As			
	Extracellular and intracellular As			
	As speciation			
FISH				
Direct behavior	Frequencies of operculum movements			
Complex behaviors	Frequencies of aggressive interactions (lunges, chases and bites)			
Physiological parameters	Change in biomass			
Arsenic content	Total bioaccumulated-As in tissue			

Table 2 Summary of the different statistical analysis used in each chapter (***Ch***) of this thesis

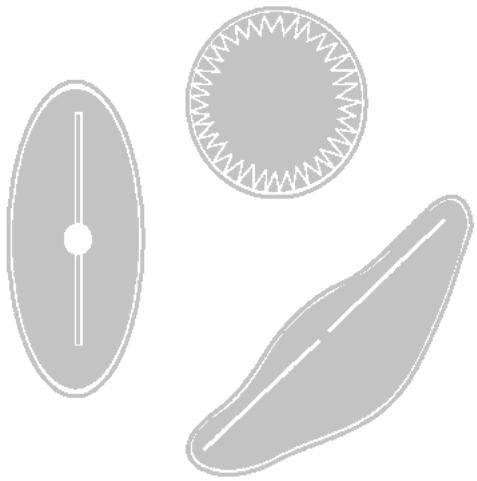
STATISTICAL ANALYSIS	Purpose of the analysis	*Ch 1*	*Ch 2*	*Ch 3*
Student's t-tests	To assess differences in specific diatom cell biovolume between treatments (*B* and *B+As*)			
One-Way ANOVAs	To assess differences in parameters measured only at the end of the experiment (chlorophyll-*a* content, arsenic bioaccumulated in biofilm and fish) and other diatom metrics, between treatments (*B* and *B+As*) only during the *As+Fish* period			
	To assess differences in diatom diversity indices (*S, H, J*) between sites (*Downstream* and *Upstream*)			
Two-Way ANOVAs	To assess differences in live bacteria between treatments (*B*, *B+As*) and in physical and chemical parameters between treatments (*noB noAs*, *As*, *B*, *B+As*), across periods (*Biofilm colonization, Arsenic* and *As+Fish)*			
	To assess differences in bacterial density, and in the arsenic accumulation (total arsenic and species) in different biofilm compartments (*rinse solution, extracellular, intracellular*) between sites (*Downstream* and *Upstream*), across time.			
Two-Way Repeated Measures ANOVA	To assess differences in biofilm photosynthetic parameters between treatments (*B*, *B+As*) and time (biofilm colonization days)			
	To assess differences in biofilm metrics and light measurements between sites (*Downstream* and *Upstream*) and time (*translocation days)*			

STATISTICAL ANALYSIS	Purpose of the analysis	*Ch 1*	*Ch 2*	*Ch 3*
Generalized Estimating Equation (GEE)	To assess differences in fish aggression (*Direct behavior*) between treatments (*C, B, A, B+A*), controlling for time (covariate)			
	To assess differences in capture efficiency and consumption (*Complex behaviors)* by fish between treatments (*C, B, A, B+A*)			
	To assess differences in the change in fish biomass (*Physical parameter)* between treatments (*C, B, A, B+A*), controlling for the total length of each fish (covariate)			
Factorial Generalized Linear model (GLM)	To assess differences in arsenic bioaccumulation in fish tissue (*Physical parameter)*			
Fitting to a 3-parameter log-normal curve	To assess changes in biofilm biomass during time (*colonization* and *experiment* period)			
Non-Metric Multidimensional Scaling plot (NMDS)	To detect possible variations of diatom community composition between sites (*Downstream* and *Upstream*), based on Bray Curtis distance			
Multi-Response Permutation Procedures (MRPP)	To test for inter-site (*Downstream* and *Upstream*) *versus* intra-site heterogeneity in diatom community structure (Zimmerman *et al.* 1985), based on Bray Curtis distance			
Redundancy Data Analysis (RDA)	To assess the effect of the environmental factors on the biological responses, using variables taken at both sites (*Downstream* and *Upstream*) and every sampling day			

3. RESULTS

CHAPTER 1

SHORT-TERM ARSENIC EXPOSURE REDUCES DIATOM CELL SIZE IN BIOFILM COMMUNITIES



Barral-Fraga L, Morin S, Rovira MD, Urrea G, Magellan K, Guasch H. (2016). Short-term arsenic exposure reduces diatom cell size in biofilm communities. *Environmental Science and Pollution Research*, 23(5): 4257-4270.

doi: http://dx.doi.org/10.1007/s11356-015-4894-8 (see Annex 1)

ABSTRACT

Arsenic (As) pollution in water has important impacts for human and ecosystem health. In freshwaters, arsenate (As^V) can be taken up by microalgae due to its similarity with phosphate molecules, being its toxicity aggravated under phosphate depletion. An experiment combining ecological and ecotoxicological descriptors was conducted to investigate the effects of As^V (130 µg L^{-1} over 13 days) on the structure and function of fluvial biofilm under phosphate-limiting conditions. We further incorporated fish (*Gambusia holbrooki*) into our experimental system, expecting fish to provide more available phosphate for algae and, consequently, protecting algae against arsenic toxicity. However, this protective role was not fully achieved. Arsenic inhibited algal growth and productivity but not that of bacteria. The diatom community was clearly affected showing a strong reduction in cell biovolume; selection for tolerant species, in particular *Achnanthidium minutissimum*; and a reduction in species richness. Our results have important implications for risk assessment, as the experimental arsenic concentration used was lower than acute toxicity criteria established by the US EPA.

1. BACKGROUND

Arsenic (As) is a widely distributed metalloid in natural ecosystems and it is considered a Priority Pollutant, being the second most common inorganic contaminant in the original National Priority List (NPL), created by the US EPA (Davis *et al.* 2001). The Aquatic Life Criteria (US EPA 2014) establishes at 340 µg L^{-1} the limit of arsenic concentration during an acute arsenic exposure in freshwaters (Criteria Maximum Concentration, CMC).

In rivers, water contaminated with arsenic have baseline concentrations ranging between 0.1 – 2.1 µg L^{-1}, with an average of 0.8 µg L^{-1} (Smedley and Kinniburgh 2002; Rahman *et al.* 2012).

A key factor in arsenic toxicity is its chemical speciation, and biological activity plays a major role in arsenic biogeochemistry (speciation, distribution and cycling) in freshwaters (Smedley and Kinniburgh 2005; Rahman *et al.* 2012). The pentavalent arsenate oxyanion (As^V) is the stable and predominant arsenic species in well oxygenated aquatic environments such as river and lake waters and oxic seawater (Smedley and Kinniburgh 2005). Little is known about As^V toxicity in algae, especially in rivers, although some studies have found that arsenic is toxic to freshwater microalgae at high concentrations, particularly at low ambient concentrations of phosphate (referred in this chapter as PO_4^{3-} or P) (e.g. Levy *et al.* 2005; Wang *et al.* 2013). Arsenate is an analog of phosphate and algae may uptake both molecules through phosphate transporters, because they share the same internalization mechanisms (Guo *et al.* 2011; Wang *et al.* 2013). It could thus be anticipated that As^V modes of toxic action might depend on phosphate availability in the environment and subsequent synthesis of phosphate transporters (Miot *et al.* 2009). In fact, aggravated arsenic toxicity has been found under phosphate depletion

in several freshwater experiments (e.g. Levy *et al.* 2005; Wang *et al.* 2013; 2014; Rodriguez Castro *et al.* 2015). In literature, laboratory experiments generally use high arsenic concentrations, and field studies are more focused on lakes. Therefore, more research on As^V toxicity and its relationship with phosphate in environmental systems is necessary, especially in rivers.

Biofilms are crucial in ecosystem functioning and have an excellent ability to degrade and transform pollutants (Mora-Gómez *et al.* 2016). In rivers, evidence of the link between metal exposure (water concentration) and metal content in biofilms has already been demonstrated, highlighting their likely effects through the trophic chain (Guasch *et al.* 2012). Biofilm complexity produces a large panel of functional and structural endpoints in both autotrophs and heterotrophs, which are often used to assess the effects of chemicals on biofilm communities (Sabater *et al.* 2007). For instance, photosynthetic parameters (Corcoll *et al.* 2012a) are early warning functional endpoints, which are usefully complemented by more structural information.

The diatom component of fluvial biofilms is among the most studied of algal organisms, due to their cosmopolitanism and predominance. Their sensitivity to many environmental factors has resulted in their wide use in water quality assessment (e.g. Coste *et al.* 2009). They respond quickly to environmental changes such as water metal contamination, as extensively documented in field and laboratory experiments. Responses of diatoms to metal pollution have generally been detected at the individual level (e.g. size, growth form, and morphological abnormalities) and/or through changes to community structure (replacement of sensitive species by tolerant ones, or decrease in species diversity) (Morin *et al.* 2012). Concerning the whole algal component, alterations of algal succession (i.e., the temporal variation in community composition during colonization, from diatoms at the beginning to cyanobacteria and filamentous green algae at the end) in biofilms exposed to metals, such as copper and zinc, have already been documented (Serra 2009; Bonet 2013).

The use of different trophic levels, e.g. fish and biofilm together, give complementary results (e.g. Griffith *et al.* 2005; Passy 2012) and may interact to modify expected toxicity (***Chapter 2***). Fish are highly sensitive to small environmental changes and arsenic is considered to be one of the most toxic elements to them (Bhattacharya *et al.* 2007). One fish species for which arsenic impacts have been demonstrated is the mosquitofish *Gambusia holbrooki* (Newman *et al.* 1989; Moeller *et al.* 2003).

In this study, we investigated the effects of short term arsenate (As^V) exposure on fluvial biofilm under the influence of fish (*Gambusia holbrooki*). Therefore, by adding fish we implemented a complex scenario in a laboratory experiment that was consequently much closer to reality than those used in classic toxicity tests. We conducted an experiment simulating a well oxygenated environment, to ensure that As^V was the dominant arsenic species, and biofilm was grown under conditions of phosphorus limitation, which is likely to lead to high arsenic toxicity.

We expected to see arsenic effects on biofilm at different scales, from diatom community structure to general algal and bacterial behavior. Effects on biofilm function and structure were anticipated, but we had no *a priori* assumptions about the intensity of effects, as both arsenate concentration and time of exposure were relatively low. Particular attention was given to diatoms, with the expectations that arsenic would cause a change in species composition and in their biovolume or cell size.

2. MATERIALS AND METHODS

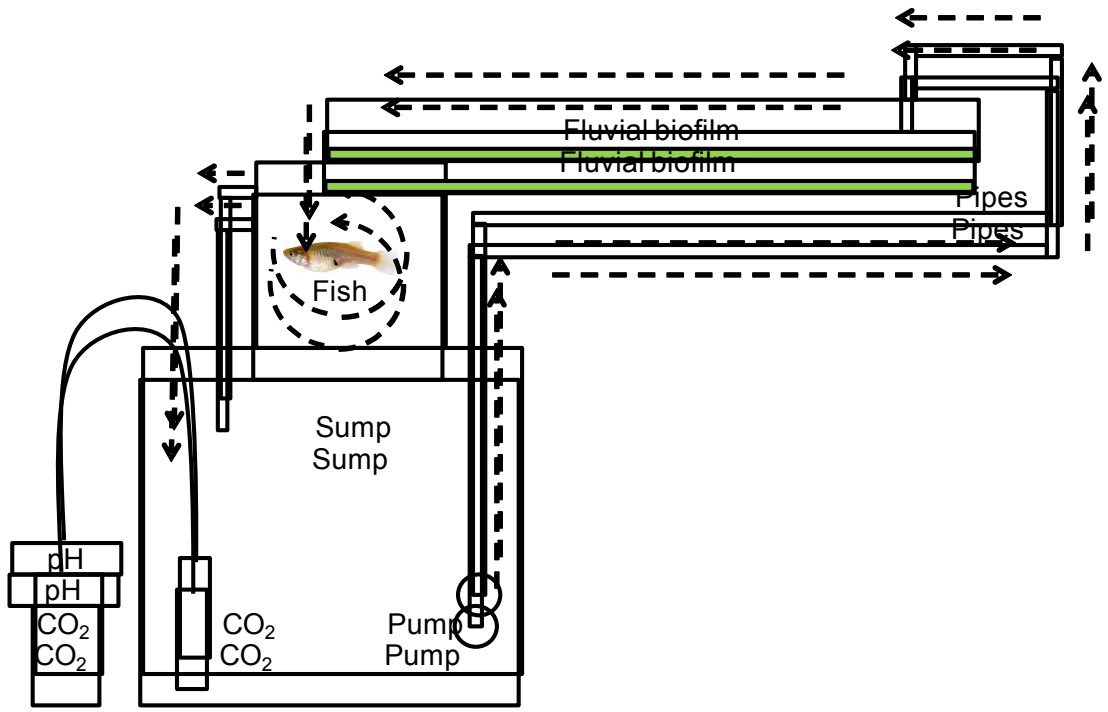
2.1. Experimental units

We constructed twelve experimental units, each consisting of a long channel (90 x 8.5 x 7.5 cm^3), as a laboratory stream, containing small (1.2 x 1.2 cm^2) and larger (7 x 7 cm^2) sandblasted glass tiles placed on the floor for biofilm colonization; a four-liter aquarium (31.5 x 11 x 31.5 cm^3) to house the fish and a sump tank (60 x 25 x 75 cm^3) filled with approximately 90 liters of water. This large volume of water ensured that changes in water chemistry were minimized. Each experimental unit was an independent system recirculating dechlorinated tap water in a constant and controlled flow rate using a hose and a submersible pump (EHEIM Universal Pumps, Germany) placed in the sump tank. Water was thus pumped from the sump tank to the head of the algal biofilm channel, passed through this channel into the 4 liter fish aquaria, where it circulated and finally returned to the sump tank (Fig. 1). The physicochemical composition of the dechlorinated tap water was characterized (see methodology in the "Water chemical sampling and analyses" section later): it is neutral water (pH 7.55±0.09), with conductivity 446.83±8.57 µS cm^{-1}, O_2 concentration 8.66±0.03 mg L^{-1} and P- PO_4^{3-} 3.70±2.93 µg L^{-1} (determined by a modified molybdenum blue method of Carvalho *et al.* 1998). Concentrations of major cations and anions dissolved in water were previously analyzed using ion-chromatography (Metrohm Ltd., Herisau Switzerland). Anions were measured using a METROSEP A SUPP 5 column and $NaHCO_3$ (84 mg L^{-1}) and Na_2CO_3 (229 mg L^{-1}) as eluents. Cations were measured using a METROSEP C 2 column and tartaric acid (2,3-dihydroxybutanedioic acid; 4 mM) and dipicolinic acid (pyridine-2,6-dicarboxylic acid; 0.75 mM) as eluents. The water contains: NO_3^- 12.73±3.58 mg L^{-1}, NO_2^- <0.01 mg L^{-1}, NH_4^+ <0.1 mg L^{-1}, SO_4^{2-} 43.74±1.03 mg L^{-1}, Ca^{2+} 33.38±1.27 mg L^{-1}, Mg^{2+} 8.43±0.35 mg L^{-1}, Na^+ 27.12±1.70 mg L^{-1} and Cl^- 46.64±0.73 mg L^{-1}.

All experimental units were housed in a room under controlled environmental conditions. Temperature was maintained at 19.5 ± 0.5 °C. Water pH was automatically controlled with a system based on CO_2 addition (JBL Proflora m630: JBL, Ludwigshafen, Germany), from 7.5 to 7.9, to provide enough inorganic carbon for algal growth. Light irradiance without heat (120W LEDs Grow Light, Lightech, Girona, Spain) was also automatically controlled, with a 12h:12h light:dark cycle.

Mosquitofish (*Gambusia holbrooki*) were collected from the Ter, Fluvià and Muga rivers (NE Spain) and transported to the laboratory where they were placed in 60 L stock aquaria (60 cm × 30 cm × 32 cm) each containing conditioned water and a filtered air supply. *Gambusia holbrooki* from all three rivers were housed together. Fish were fed to satiation once per day with commercial food flakes or defrosted frozen bloodworms (*Chironomus* spp.) and were able to acclimate to captivity conditions for at least 6 months, with a further month to acclimate to experiment-specific environmental parameters (e.g. temperature). During the experiments, fish and biofilms were not together but separated into different compartments of the experimental units: fish were placed in the four-liter aquarium, while biofilms were grown in the channels. This ensured that fish could not graze biofilms. Fish were also fed to satiation during the experiment with the commercial frozen bloodworms (*Chironomus* spp.).

a)



b)

c)

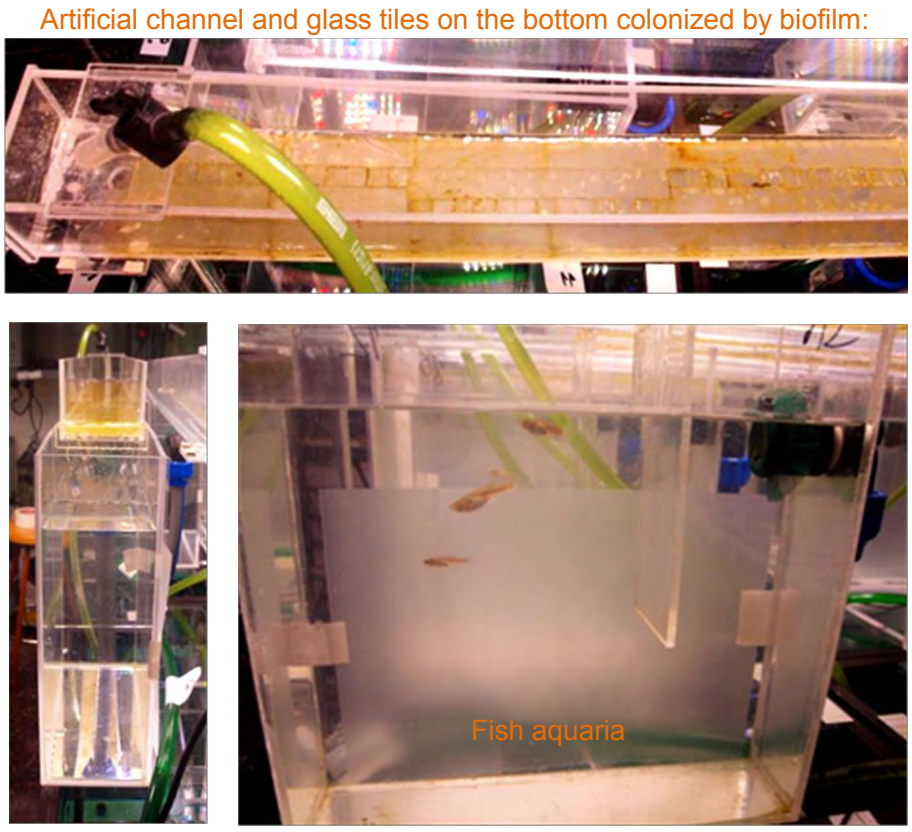


Figure 1 Experimental unit: a) schematic diagram (the dashed arrows show the direction of water flow); b) and c) are pictures of the experimental units and the detail of the different parts (see main text for details).

2.2. Experimental design

Our experimental design consisted of three replicates of each of four different treatments. Treatments were: *noB noAs*, arsenic (with arsenic only), *B* (with biofilm only) and *B+As* (with both biofilm and arsenic) (Fig. 2). First, natural biofilm inoculum was added to six of the experimental units and allowed to grow and colonize the sandblasted glass tiles (*Biofilm colonization period*). After colonization (20 days), As^V (130 μg L^{-1}) was added to six of the experimental units (*As period*). This time lag was expected to influence dissolved arsenic concentration in the *B+As* treatment due to uptake and/or adsorption. Four days later, four fish (1 male, 3 females) were added to each experimental unit, such that each contained the same fish biomass (*As+Fish period*). The experiment ended after 33 days of biofilm colonization. Thus, biofilms were exposed to As^V for 13 days, and fish exposure lasted for 9 days (Fig. 2).

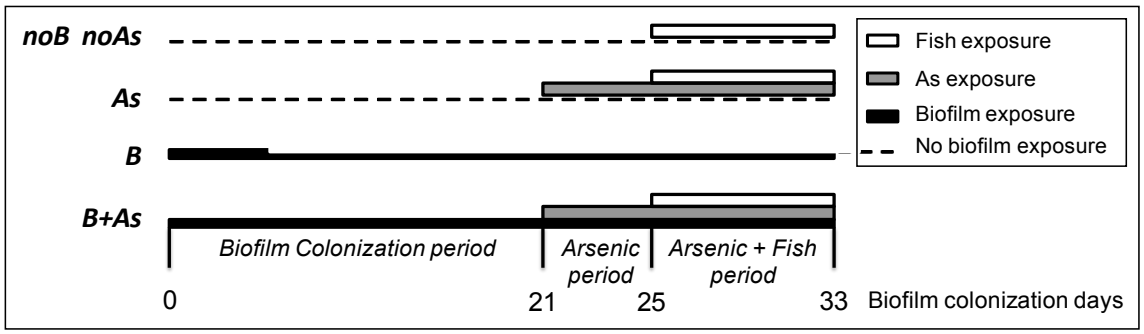


Figure 2 Timeline (biofilm colonization days) of this experimental study. White, gray and black rectangles represent the exposure time of fish, arsenic and fluvial biofilm respectively, in the experimental units. Black dotted lines represent absence of biofilm in the experimental units. Time was divided into three parts: *Biofilm Colonization period, Arsenic period, Arsenic+Fish period* (see main text for details).

Biofilm colonization period

Biofilm was colonized on sandblasted glass tiles (1.44 cm^2 and 49 cm^2), placed at the bottom of each channel. Several rocks were chosen at random from the upstream zone of the Llémena Stream (NE Spain), a small calcareous tributary of the Ter River that had minimal human impact. Rocks were transported to the laboratory in boxes filled with river water that were placed inside a portable fridge to ensure biofilms were always wet and fresh. Once in the laboratory, all rock surfaces were scraped and, then scraped biofilm was added as an inoculum to the channel (artificial stream) of each experimental unit (the same volume in each one) twice per week during the three-week colonization period (from biofilm colonization day 1 to 20). Once per week, water levels were adjusted and 10 μg L^{-1} of phosphate (KH_2PO_4, Merk, Darmstadt, Germany) were added to reproduce phosphate limiting conditions for algal growth (Dodds *et al.* 1998). The use of clean artificial substrates, instead of already colonized rocks, allowed monitoring of biofilm colonization and algal succession in experimental conditions.

Biofilm development was controlled regularly, measuring the *Fo* parameter (the minimal fluorescence yield of a dark adapted cell) that gives a fluorescence proportional to the biofilm chlorophyll-a concentration. This parameter was obtained by using the PhytoPAM (Pulse Amplitude Modulated) fluorometer (HEINZ WALZ, Effeltrich, Germany), as detailed in the "Biofilm measurements" section.

Arsenic period

After 20 days of colonization, young biofilms, but close to maturity, had developed indicating the best time to begin the arsenic exposure while avoiding senescence at the end of the experiment. Thus, on biofilm colonization day 21, As^V solution as $NaH_2AsO_4.7H_2O$ (Merk, Darmstadt, Germany) was added to six of the experimental units without (*As* treatment) and with (*B+As* treatment) biofilm, to reach the nominal concentration of 130 µg As^V L^{-1}. After arsenic addition the only addition of water was to replace water lost through evaporation. Therefore, the *Arsenic period* began on biofilm colonization day 21 and ended on day 24.

As it was expected that biofilm would retain arsenic, the arsenic was added before adding fish in order to check the influence of this retention on the reduction of exposure to fish.

As+Fish period

On day 25, all fish were weighed, total length was measured, and four fish were added to each experimental unit. Different sized females were used primarily to allow identification of individuals within an aquarium so any overlap in sizes between aquaria was tolerated.

2.3. Water chemical sampling and analyses

Physical and chemical parameters (water temperature, dissolved oxygen, conductivity and pH) were measured with appropriate probes during the whole experimental period (33 days). Dissolved oxygen and conductivity were measured 6-10 times (HQ Portable Meters, HQ40d18, HACH Company), whereas phosphate and total dissolved arsenic were measured 10 and 7 times respectively for each experimental unit.

Triplicate water samples (10 mL) were taken for chemical analyses from each experimental unit 10 times during the experiment. Water was filtered with GF/F Glass Microfiber Filters (Whatman, 0.7 µm of pore size) for phosphorus determination, but for total dissolved arsenic water samples were filtered with 0.2 µm nylon membrane filters (Whatman) and immediately acidified with 1% of HNO_3 (65% suprapure, Merck). All water samples were frozen (at -20 °C) until analysis.

Inorganic phosphate (iP) concentration was determined by a modified molybdenum blue method (Carvalho *et al.* 1998) to avoid arsenate interference. Briefly, 10 mL of the sample were pipetted into a digestion tube and 2 mL of L-cysteine (5% w/v in 0.6 M HCl) were added. The

tube was tightly capped and incubated for 5 min at 80ºC to allow complete reduction of arsenate into arsenite. The solution was cooled to ambient temperature (25 °C) and then inorganic phosphate was determined with 0.5 mL of ascorbic acid (5% w/v in deionized water), 1 mL of acetone and 2 mL of mixed reagent (50 mL of sulfuric acid 20%, 5 mL of antimony potassium tartrate, 15 mL of ammonium molybdate and made up to 100 mL with Milli-Q water). Absorbance was quantified at 875 nm.

2.4. Biofilm measurements

Chlorophyll-a fluorescence-related endpoints

Photosynthetic activity and algal biomass of the biofilm were measured on days 7, 10, 14, 17, 21, 25, 26, 28, 31 and 33 using the PhytoPAM (Pulse Amplitude Modulated) fluorometer (HEINZ WALZ, Effeltrich, Germany) connected to an Emitter Detector Fiberoptics Unit (PHYTO-EDF) and "PhytoWin" software. PAM fluorometry is a rapid, non-invasive and reliable method to assess photosynthesis performance, and has been found to be the most sensitive tool for the rapid detection of harmful compounds (Corcoll *et al.* 2012a). Five replicates (small colonized sandblasted glass tiles) were used from each experimental unit (*B* and *B+As* treatments) each time. Temperature (19 ºC) and distance between light emitting diode and samples (8mm) were kept constant for all the measurements. First, measurements of dark adapted samples were done at the end of the darkness cycle. A saturation pulse was applied and the minimum fluorescence yield was obtained. According to Corcoll *et al.* (2012a), the minimal fluorescence yield of a dark adapted cell (F_0) is proportional to its chlorophyll-a concentration. Thus, it can be used as an estimation of algal biomass. The maximum PSII quantum yield (*Ymax)* was also obtained during the saturation pulse performed under dark conditions. This parameter is defined as a measure of the photosynthetic capacity of the community (Corcoll *et al.* 2012a). Thereafter, light adaptation of the samples was carried out for 15 minutes for light measurements. Actinic light provided by the instrument was used. One saturation pulse was applied and the effective PSII quantum yield (Photosynthetic efficiency, *Yeff*) was obtained. Effective PSII quantum yield is defined as a measure of the photosynthetic efficiency of the community (Corcoll *et al.* 2012a). After all measures, colonized sandblasted glass substrata were returned into the experimental units channels.

Bacterial abundance

The double staining Live/Dead BacLight Bacterial Viability Kit (Molecular Probes) was used to measure the abundance of live and dead bacteria in the biofilm samples. Four times during the experiment, small colonized sandblasted glass tiles were collected into autoclaved glass vials, resuspended and then diluted in autoclaved Milli-Q water. All cells were firstly individualized by sonication (less than one minute to avoid damaging cell membranes) and stained using a mixture of 3.34mM SYTO® 9. Then, only dead cells (those with cell membranes

damaged during the experiment) were stained by 20mM propidium iodide (Freese *et al.* 2006). After 30 minutes in dark conditions, each sample was filtered through a 0.2 µm black polycarbonate filter (Nuclepore, Whatman). Twenty random microscopy fields were counted for each sample (filter) using epifluorescence microscopy at a magnification of 1000x in immersion oil (Nikon E600, Tokyo, Japan). Data are expressed as live bacteria (cell cm^{-2}).

Benthic chlorophyll-a

On the last day of the experiment (after 13 days of biofilm arsenic exposure), small and colonized sandblasted glass tiles were collected from each channel into falcon tubes, immediately frozen in liquid nitrogen and stored at -80ºC until chlorophyll-a extraction. The chlorophyll-a content was extracted with 90% acetone for 12 h. Sonication (Ultrasonic bath, J.P Selecta) for 2 minutes improved the pigment extraction and chlorophyll-a concentration was subsequently estimated from spectrophotometric measurements (spectrophotometer UV-1800, Shimadzu), following the method described in Jeffrey and Humphrey (1975). Since the biofilm was colonized on the surface of the tile, when the tile was submerged in 90% acetone for chlorophyll-a extraction and then sonicated, chlorophyll-a from the whole biofilm colonized on the tile was obtained.

Diatom community identification and metrics

Diatoms were collected from 1 small colonized sand blasted glass substratum from each channel at the end of the experiment. Biofilm was immediately resuspended and conserved in a glass vial with 4.5 mL of Milli-Q water and 0.5 mL of 40% formaldehyde. Then, samples were digested with 10 mL of hydrogen peroxide (30% H_2O_2) to eliminate organic matter and obtain clean frustules according to Leira and Sabater (2005). Frustules were then washed with distilled water, dehydrated on cover glasses and finally mounted on permanent slides using Naphrax (Refractive index 1.74; Brunel LTD, UK). All these steps were carefully performed with controlled volumes, to allow a final quantitative assessment of diatom densities. Up to 600 diatom valves per slide were counted and identified to assess species richness and diversity in our samples. Random transects were scanned under a light microscope (Nikon E600, Tokyo, Japan) using Nomarski differential interference contrast optics at a magnification of 1000x. Identification mainly followed Krammer and Lange-Bertalot (1986–1991), and recent nomenclatural updates. Diatom species relative abundance and density were calculated, as well as the species richness (*S*), Shannon-Wiener index of diversity (*H*) and species evenness (*J*). Calculations for *H* and *J* were performed using the following equations:

$$H = -\sum_{i=1}^{S} P_i \ln P_i \quad ; \quad J = \frac{H}{H_{max}} = \frac{-\sum_{i=1}^{S} P_i \ln P_i}{\ln S},$$

where P_i is the proportional abundance of the *i*th species and S is the total number of species present in the community (species richness).

Diatom biovolume determination

Diatom specific biovolume was determined using light microscopy with Nomarski differential interference contrast optics at a magnification of 1000x and following a set of geometrical shapes proposed by Hillebrand *et al.* (1999). Cell size (or cell biovolume) was calculated by measuring different dimensions (length, width, diameter and some heights) of 25 randomly selected valves per species, as far as possible, and using equations from a set of geometrical shapes proposed by Hillebrand *et al.* (1999). Total species biovolume was then calculated.

In addition, since theoretical cell biovolume data has been used in several studies, our measured cell biovolumes were compared with the theoretical ones (http://hydrobio-dce.irstea.fr/cours-deau/diatomees/) corresponding to each species.

2.5. Arsenic measurements

The level of arsenic in the circulating system was measured 7 times during the whole exposure period: 4 times before adding fish and 3 times after adding fish. For biofilm samples, total arsenic accumulation was measured at the end of the exposure (6 samples/channel). For all analyses, the detection limit was 0.08 µg L^{-1}; Rhodium (Rh) was used as the internal standard and the accuracy of the analytical method was checked periodically using a certified water reference (SPS-SW2 Batch 113, Oslo, Norway).

Total dissolved arsenic concentration

Total dissolved arsenic concentration (µg L^{-1}) was analysed by inductively coupled plasma mass spectroscopy (ICP-MS 7500c Agilent Technologies, Inc. Wilmington, DE).

Total arsenic accumulation in biofilm

Total arsenic accumulation in biofilm was analyzed in triplicate for treatments *B* and *B+As* (using large sand blasted glass substrata). Colonized glass substrates were collected at the end of the experiment, placed on filter paper to remove excess water, and immediately frozen before analysis. Then, biofilm was freeze-dried, weighed and digested using 4 ml of concentrated HNO_3 (65% suprapure, Merck, Germany) in a high performance microwave digestion unit (Milestone, Ethos Sel). They were then diluted to 15 mL with milli-Q water and the subsequent liquid samples were treated as dissolved metal water samples. Total dissolved arsenic concentration was measured by ICP-MS (7500c Agilent Technologies, Inc. Wilmington, DE).

2.6. Data analysis

Prior to statistical analyses, some variables had to be log-transformed (from water physical and chemical data, only phosphate concentration and total dissolved arsenic were log-transformed; from biological data, live bacteria and bioaccumulated arsenic on biofilm; and photosynthetic parameters were also log-transformed), or log(x+1) transformed (diatom relative frequencies) to reduce skewed distributions and fix heteroscedasticity. For chemical measurements, half of the detection limit was used for data treatment when the value obtained was below the detection limit (Helsel 1990).

Most data were taken several times during the experiment. Significant differences between treatments and time together were analyzed. Two-Way ANOVAs were applied to physical and chemical data, where the *Time* variable was categorized in three periods: *Biofilm colonization, arsenic* and *As+Fish*. Biofilm photosynthetic parameters were analyzed by Two-Way Repeated Measures ANOVA, where the *Time* variable (expressed in biofilm colonization days) was the within-subject continuous variable, and *Treatment* (biofilm treatment, *B*, *versus* biofilm with arsenic exposure, *B+As*) was the between-subject variable. Finally, post-hoc Bonferroni's tests were applied to check exactly where significant differences were found.

For data taken only at the end of the experiment (chlorophyll-*a* content, arsenic bioaccumulated in biofilm and fish) and diatom metrics, One-Way ANOVAs were performed to analyze significant differences between treatments. For diatom species relative abundance, only the species that represented more than 0.5% of the total relative abundance were considered in the ANOVA analysis. For total diatom cell biovolume, One-Way ANOVA was also performed. However, specific diatom cell biovolume were analyzed with Student's t-tests, since heteroscedasticity was not reduced with the log-transformation. Student's t-test is analogous to the One-Way ANOVA with two treatments, but it allows to obtain results even in case of heteroscedasticity. Statistical significance for all the ANOVA's and Student's t-tests was set at $p \leq 0.05$; while marginal significance was set at $0.05 < p \leq 0.1$. Correlation analysis was done to compare measured and theoretical diatom cell biovolume data.

SPSS software (version 15.0) was used for statistical analyses. Boxplots for the description of the diatom cells biovolume, as well as the correlation analysis between measured and theoretical data, were done with Microsoft Excel 2010 software. The graphics for the photosynthetic parameters and physicochemical variables were developed using Sigmaplot software (version 11.0).

3. RESULTS

3.1. Physico-chemical and bioaccumulation data

A time effect was observed with water chemistry and arsenic also had a significant effect, especially after fish addition. Physico-chemical data, as well as the ANOVAs' results and comparison per pairs, are summarized in Table 1. Water conductivity slightly decreased over the whole experiment (time effect), and was lower in the experimental units with biofilms (*B* and *B+As*; mean values of 427.19 ± 6.39 µS cm^{-1} over the experiment) than in those without biofilm (*noB noAs* and *As*; 441.75 ± 7.48 µS cm^{-1}). In general, lower values were found in the *B* treatment than in the *B+As* treatment. For dissolved oxygen, a general decrease was observed during the *As+Fish period*, being significantly lower (p<0.001) in biofilm exposed to arsenic than in biofilm without arsenic. On the other hand, a significant increase in phosphate concentration in water was observed except in the arsenic treatment at the end of the experiment (p<0.001), during the *As+Fish period*. Also in that period, arsenic accumulation in the biota reflected exposure (Table 2), with higher arsenic content in biofilm (p<0.001) and fish (p=0.012).

3.2. Biofilm measurements

Bacteria

Live bacteria (cell cm^{-2}) increased in both biofilm treatments over the experiment (p=0.015) from a mean of $4.09x10^6$ ± $1.25x10^6$ cell cm^{-2} during the *Biofilm colonization period* to a mean of $13.17x10^6$ ± $8.23x10^6$ cell cm^{-2} in the *As+Fish period* (Table 2). No significant difference was observed between treatments *B vs*. *B+As*.

Table 1 Water physical and chemical data, with statistical results. Water physical and chemical data are represented by the mean ± standard deviation, and sample size (n). Statistical results (F and *p*) for effects on Time (degrees of freedom, d*f*=2) and Treatment (d*f*=3) were achieved by Two-Way ANOVA and Bonferroni's test (different letters indicate significant differences between sampling time or treatments at $p \leq 0.05$). bdl: below detection limit. *Stars indicate marginal significance ($0.05 < p \leq 0.1$).

Time period	Treatment	Conductivity (µS cm^{-1})	O_2 (mg L^{-1})	PO_4^{3-} (µg L^{-1} - P)	Total As (µg L^{-1})
Biofilm colonization	***B***	444.00 ± 3.10 (n=6)	8.66 ± 0.04 (n=6)	5.83 ± 5.46 (n=6)	
	B+As	448.50 ± 7.34 (n=6)	8.62 ± 0.02 (n=6)	bdl (n=6)	
As	***noB noAs***	439.00 ± 2.92 (n=9)	8.80 ± 0.09 (n=9)	bdl (n=3)	1.98 ± 0.12 (n=12)
	As	435.78 ± 4.71 (n=9)	8.77 ± 0.03 (n=9)	bdl (n=3)	124.89 ± 2.43 (n=12)
	B	424.50 ± 2.46 (n=9)	8.82 ± 0.23 (n=9)	2.51 ± 0.02 (n=3)	2.01 ± 0.15 (n=12)
	B+As	429.40 ± 1.51 (n=9)	8.56 ± 0.08 (n=9)	3.07 ± 0.99 (n=3)	121.00 ± 4.06 (n=12)
As + fish	***noB noAs***	446.44 ± 9.68 (n=9)	8.58 ± 0.10 (n=9)	12.11 ± 4.10 (n=3)	1.92 ± 0.09 (n=9)
	As	445.78 ± 5.33 (n=9)	8.50 ± 0.06 (n=9)	3.18 ± 1.17 (n=3)	127.96 ± 5.55 (n=9)
	B	419.40 ± 1.94 (n=9)	8.69 ± 0.27 (n=9)	12.28 ± 3.34 (n=3)	1.89 ± 0.11 (n=9)
	B+As	435.30 ± 3.28 (n=9)	8.34 ± 0.12 (n=9)	15.96 ± 4.14 (n=3)	124.20 ± 2.64 (n=9)
Time effects	ANOVA	F=78.177, *p*<0.001	F=21.076, *p*<0.001	F=34.690, *p*<0.001	F=0.801, *p*=0.374
	Biofilm colonization	a	a	a	
	As	b	a	a	a
	As + fish	c	b	b	a
Treatment effects	ANOVA	F=66.824, *p*<0.001	F=11.293, *p*<0.001	F=5.226, *p*=0.006	F=48006.691, *p*<0.001
	noB noAs	a	a	a	a
	As	a,c	a	b*	b
	B	b	a	a	a
	B+As	c	b	a*	b

Table 2 Biological data with statistical results. Biological data are represented by the mean ± standard deviation, and sample size (n). Statistical results (F and *p*) for effects on Time (degrees of freedom, d*f*=2) and Treatment (d*f*=1) were achieved by Two-Way ANOVA (for Live bacteria data) and One-Way ANOVA (for Chl-a, arsenic biofilm and arsenic fish data). Bonferroni's tests were also carried out (different letters indicate significant differences between sampling time or treatments at $p \leq 0.05$).

Time period	Treatment	Live bacteria ($x10^6$ cell cm^{-2})	Chl-a ($\mu g\ cm^{-2}$)	As biofilm ($\mu g\ g^{-1}$)	As fish ($\mu g\ g^{-1}$)
Biofilm colonization	***B***	5.12± 0.50 (n=3)			
	B+As	3.05 ± 0.69 (n=3)			
As	***B***	9.81 ± 7.52 (n=3)			
	B+As	5.83 ± 2.04 (n=3)			
As + fish	***B***	12.23 ± 8.47 (n=6)	40.61 ± 7.56 (n=3)	3.251 ± 0.21 (n=6)	470.95 ± 61.38 (n=3)
	B + As	14.11 ± 8.67 (n=6)	22.72 ± 8.64 (n=3)	79.59 ± 9.39 (n=6)	758.09 ± 95.32 (n=3)
Time effects	ANOVA	F=4.980, *p*=0.019			
	Biofilm colonization	a			
	As	a,b			
	As + fish	b			
Treatment effects (*B vs. B+As*)	ANOVA	F=0.623, *p*=0.440	F=7.282, *p*=0.054	F=3297.04, *p*<0.001	F=19.243, *p*=0.012

Chlorophyll-a fluorescence measurements and Chlorophyll-a content

Minimum fluorescence yield (F_0) increased over time and showed significant differences between treatments *B* and *B+As* (Fig. 3, Table 3) during the *As+Fish period*, revealing a significant inhibition of algal biofilm growth from day 25 to day 33 (Fig. 3). Chlorophyll-a concentration showed a similar result (Table 2).

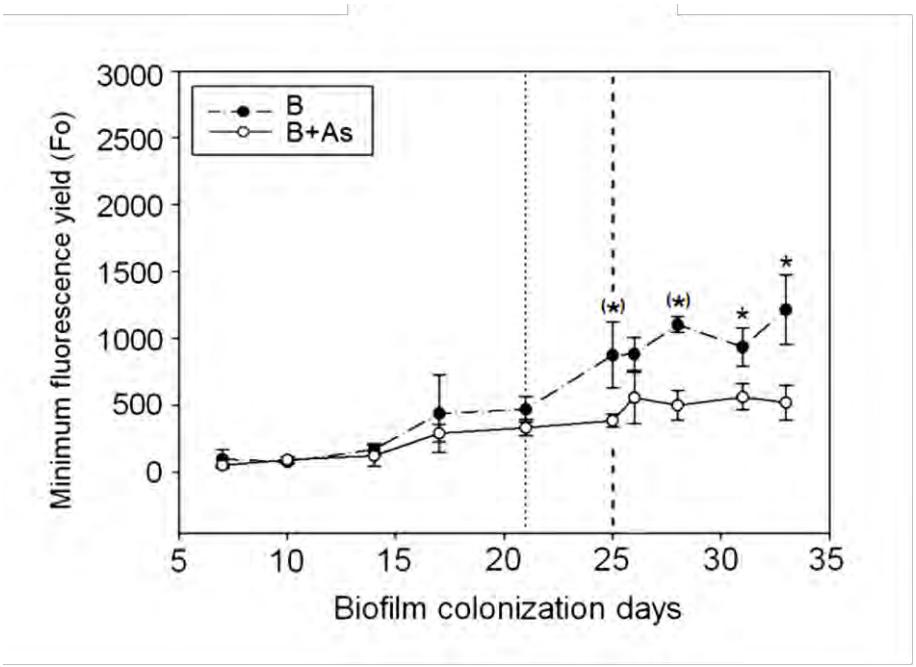


Figure 3 Biofilm growth: Evolution of Minimum fluorescence yield (F_0) during the biofilm colonization days until the end of the experiment in the different treatments (*B*, biofilm without arsenic exposure; *B+As*, biofilm with arsenic exposure). Vertical lines indicate arsenic addition (on biofilm colonization day 21) and fish addition (on biofilm colonization day 25). Stars indicate significant differences (at $p \leq 0.05$) between treatments for each day. Stars in brackets indicate marginal significance ($0.05 < p \leq 0.1$).

Arsenic also affected algal succession and photosynthetic parameters of the different groups of algae and cyanobacteria. In the *B* treatment, diatoms and cyanobacteria increased in biomass during the 4 first weeks, then decreased, and were followed by a progressive growth of filamentous green algae. In contrast, green algae did not grow with arsenic (Fig. 4). Significant differences in the maximum PSII quantum yield (*Ymax*) between treatments were found. *Ymax (diatoms)* was lower during the whole period of arsenic exposure, in contrast to *Ymax (cyanobacteria)* and *Ymax (general)* that showed more scattered results (Fig. 5). The effective PSII quantum yield (*Yeff*) also showed significant differences, except in diatoms (*Yeff diatoms*), at the end of the experiment (Fig. 5, Table 3).

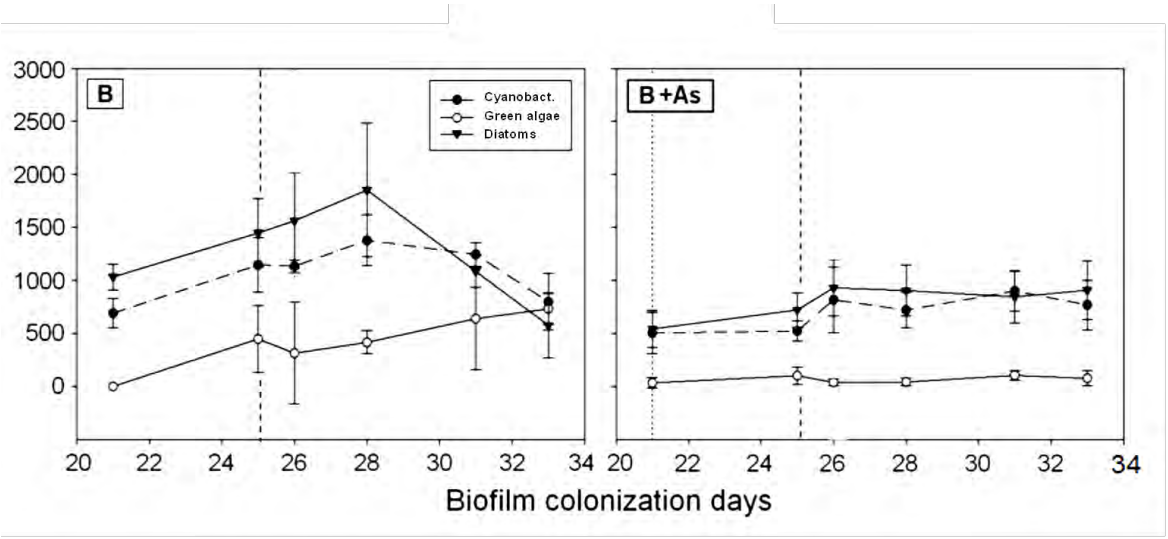


Figure 4 Algal succession: Evolution of Minimum fluorescence yield (F_0) of each algal group (Cyanobacteria, Green algae and Diatoms) during the arsenic exposure and until the end of the experiment, compared between treatments (*B vs. B+As*). Vertical lines indicate arsenic addition (biofilm colonization day 21) and fish addition (biofilm colonization day 25).

Table 3 Statistical results of biofilm photosynthetic parameters. Two-Way Repeated Measures ANOVA (F and *p*) of photosynthetic parameters was performed for all algae (general), cyanobacteria, filamentous green algae and diatoms to analyze statistical differences in time (sample size, n=6; degrees of freedom, d*f*=5) and between treatments (*B vs. B+As;* n=2; d*f*=1) at $p \leq 0.05$. F_0 parameters represent the minimal fluorescence yield of a dark adapted cell, *Ymax* parameters represent the photosynthetic capacity of the community, and *Yeff* parameters represent the photosynthetic efficiency.

Photosynthetic parameters	Time		Treatment (*B vs. B+As*)	
	F	*p*	F	*p*
Fo (general)	14.351	<0.001	27.910	0.006
Fo (cyanobacteria)	5.157	0.003	12.602	0.024
Fo (green algae)	11.103	<0.001	2.170	0.215
Fo (diatoms)	5.400	0.003	4.220	0.109
Ymax (general)	6.581	0.001	66.217	0.001
Ymax (diatoms)	1.509	0.231	127.755	<0.001
Ymax (cyanobacteria)	1.803	0.158	9.500	0.037
Yeff (general)	2.313	0.082	40.863	0.003
Yeff (diatoms)	0.276	0.921	1.290	0.320
Yeff (cyanobacteria)	0.961	0.465	75.072	0.001

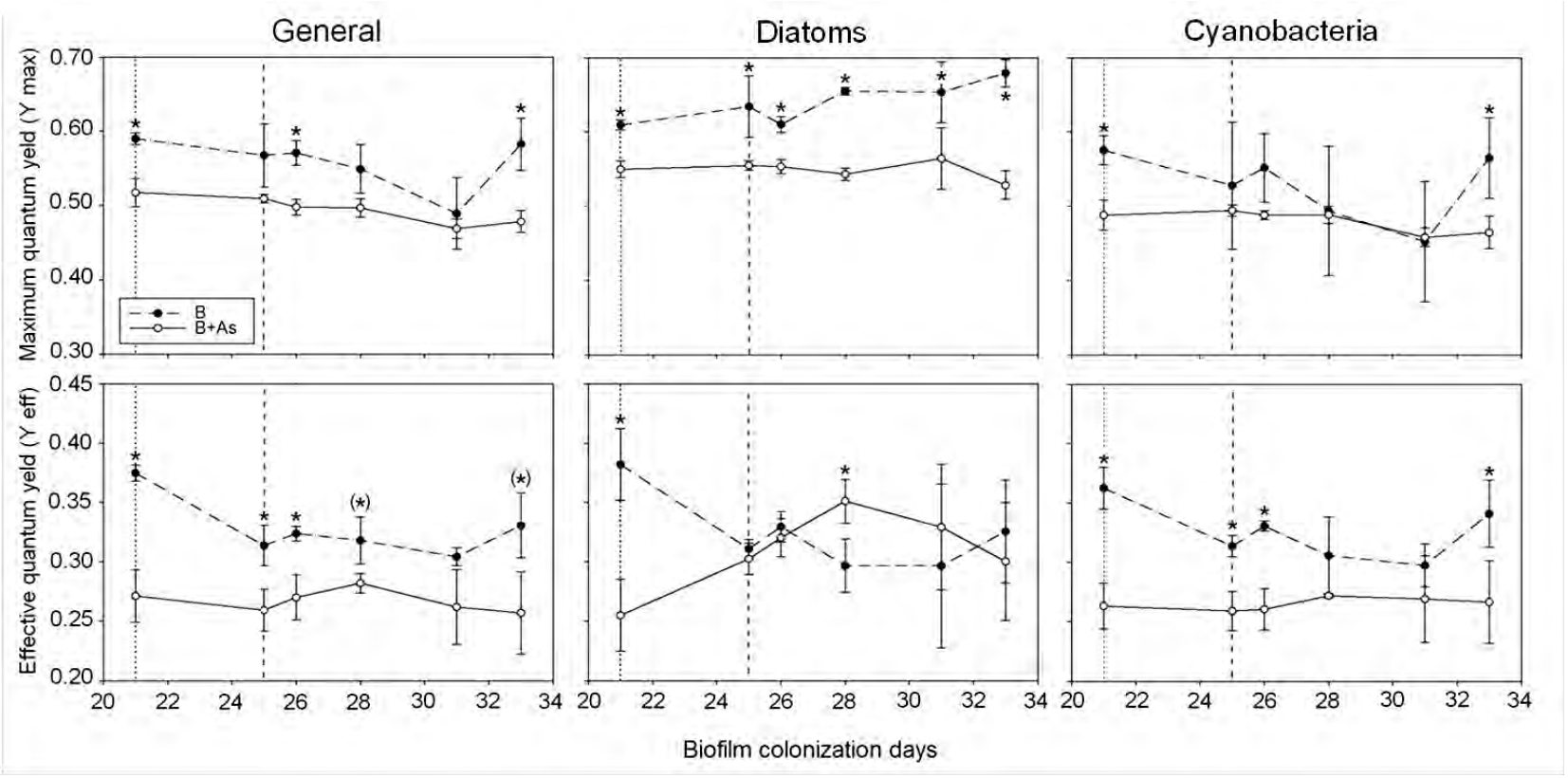


Figure 5 Evolution of Maximum quantum yield (*Ymax*) and Effective quantum yield (*Yeff*) of the algal groups together (General) and individual groups (Diatoms and Cyanobacteria) from the arsenic addition event until the end of the experiment. Vertical lines indicate arsenic addition (biofilm colonization day 21) and fish addition (biofilm colonization day 25). Statistical comparison between treatments (*B vs. B+As*) was done: stars indicate significant differences ($p \leq 0.05$) between treatments in each day; stars in brackets indicate marginal significance ($0.05 < p \leq 0.1$).

Diatom community identification and metrics

We identified 52 diatom taxa (Table 4), of which *Achnanthidium minutissimum* (Kützing) Czarnecki was the most abundant species, representing almost the 77% of the total abundance of diatoms (75% in *B* treatment and almost 79% in *B+As*). In general, the relative abundances of other species decreased when they were exposed to arsenic. Significant decreases were found in *Amphipleura pellucida* Kützing (p=0.007) and *Nitzschia dissipata* (Kützing) Grunow ssp. *dissipata* (p=0.004) whereas a significant proportion of diatom species (30%) increased in cell numbers, highlighting some *Fragilariaceae*, in particular *Ulnaria ulna* (Nitzsch) Compère (p=0.092).

Furthermore, arsenic effects on diatom species richness were marginally significant (p=0.051, Table 5).

Table 4 List of the all diatom taxa found at the end of the experiment

Achnanthidium minutissimum (Kützing) Czarnecki
Achnanthidium subatomoides (Hustedt) Monnier, Lange-Bertalot et Ector
Amphipleura pellucida Kützing
Amphora aff. *veneta* (Kützing)
Amphora inariensis Krammer
Amphora pediculus (Kützing) Grunow
Aneumastus stroesei (Ostrup) Mann & Stickle in Round Crawford & Mann
Caloneis sp.
Cocconeis placentula Ehrenberg var. *placentula*
Cyclotella meneghiniana Kützing
Cymbella affinis Kützing var. *affinis*
Cymbella cistula (Ehrenberg) Kirchner
Cymbopleura amphicephala Krammer
Denticula tenuis Kützing
Diploneis sp.
Encyonema minutum (Hilse in Rabhenhorst) D.G. Mann in Round Crawford & Mann
Encyonema prostratum (Berkeley) Kützing
Encyonopsis falaisensis (Grunow) Krammer
Encyonopsis microcephala (Grunow) Krammer
Eolimna minima (Grunow) Lange-Bertalot
Fragilaria capucina Desmazières var. *capucina*
Fragilaria capucina Desmazières var. *vaucheriae* (Kützing) Lange-Bertalot
Fragilaria gracilis Østrup
Fragilaria mesolepta Rabenhorst
Frustulia vulgaris (Thwaites) De Toni
Gomphonema lateripunctatum Reichardt & Lange-Bertalot
Gomphonema parvulum (Kützing) Kützing var. *parvulum* f. *parvulum*

Gomphonema truncatum Ehrenberg
Gyrosigma acuminatum (Kützing) Rabenhorst
Halamphora veneta (Kützing) Levkov
Mayamaea atomus (Kützing) Lange-Bertalot var. *atomus*
Melosira varians Agardh
Navicula aff. *saprophila* Lange-Bertalot & Bonik
Navicula capitatoradiata Germain
Navicula cryptotenella Lange-Bertalot
Navicula gregaria Donkin
Navicula menisculus Schumann var. *menisculus*
Navicula reichardtiana Lange-Bertalot var. *reichardtiana*
Navicula tripunctata (O.F.Müller) Bory
Nitzschia amphibia Grunow f. *amphibia*
Nitzschia dissipata (Kützing) Grunow ssp. *dissipata*
Nitzschia fonticola Grunow in Van Heurck
Nitzschia palea (Kützing) W.Smith
Nitzschia recta Hantzsch in Rabenhorst
Planothidium lanceolatum (Brebisson ex Kützing) Lange-Bertalot
Rhoicosphenia abbreviata (C.Agardh) Lange-Bertalot
Sellaphora stroemii (Hustedt) Kobayasi in Mayama Idei Osada & Nagumo
Staurosira brevistriata (Grunow) Grunow
Staurosira construens Ehrenberg
Staurosira mutabilis (Wm Smith) Grunow
Staurosira venter (Ehrenberg) Cleve & Moeller
Ulnaria biceps (Kützing) Compère
Ulnaria capitata (Ehrenberg) Compère
Ulnaria ulna (Nitzsch) Compère

Diatom biovolume determination

Arsenic clearly reduced diatom average cell biovolume (p=0.003, see Table 5). Besides the global decrease in cell size, individual cell biovolume (or cell size) in some species, such as *Nitzschia fonticola,* was also reduced with arsenic exposure (p=0.066, Fig. 6a), although this result must be treated with caution because of data heteroscedasticity. There was a general trend in biovolume decrease (measured as total biovolume per sample surface unit, Table 5), which was statistically significant in one case, *Nitzschia dissipata* (p=0.040), and marginally significant in three cases, *Amphipleura pellucida* (p=0.051), *Nitzschia fonticola* (p=0.079) and *Nitzschia palea* (Kützing) W.Smith (p=0.073). In contrast, the biovolume of some *Fragilaria* species, such as *Fragilaria capucina* Desmazières var*. capucina* and *Fragilaria mesolepta* Rabenhorst, increased under arsenic exposure due to greater cell size and/or higher cell numbers in the arsenic treatment (Fig. 6b).

Table 5 Diatom metrics and biovolume data, with statistical results. Data are represented by the mean ± standard deviation. Three replicate samples were used for each datum (n=3). One-Way ANOVA was performed to analyze statistical differences between treatments (*B vs. B+A;* d*f*=1) at $p \leq 0.05$.

Treatment		Species Richness (*S*)	Shannon Diversity Index *(H)*	Species Eveness *(J)*	Density (cell cm^{-2})	Mean cell biovolume (μm^3)	Total diatom biovolume ($\mu m^3\ cm^{-2}$)
B		32.00 ± 4.36	1.19 ± 0.16	0.34 ± 0.04	$73.67 \times 10^6 \pm 28.36 \times 10^6$	153.41 ± 10.20	$2.20 \times 10^{12} \pm 1.40 \times 10^{12}$
B+As		24.67 ± 1.53	0.98 ± 0.15	0.31 ± 0.05	$70.67 \times 10^6 \pm 21.57 \times 10^6$	84.43 ± 15.17	$1.15 \times 10^{12} \pm 0.70 \times 10^{12}$
One-Way ANOVA	F	7.563	2.580	1.246	0.025	42.724	1.349
	p	0.051	0.183	0.327	0.882	0.003	0.310

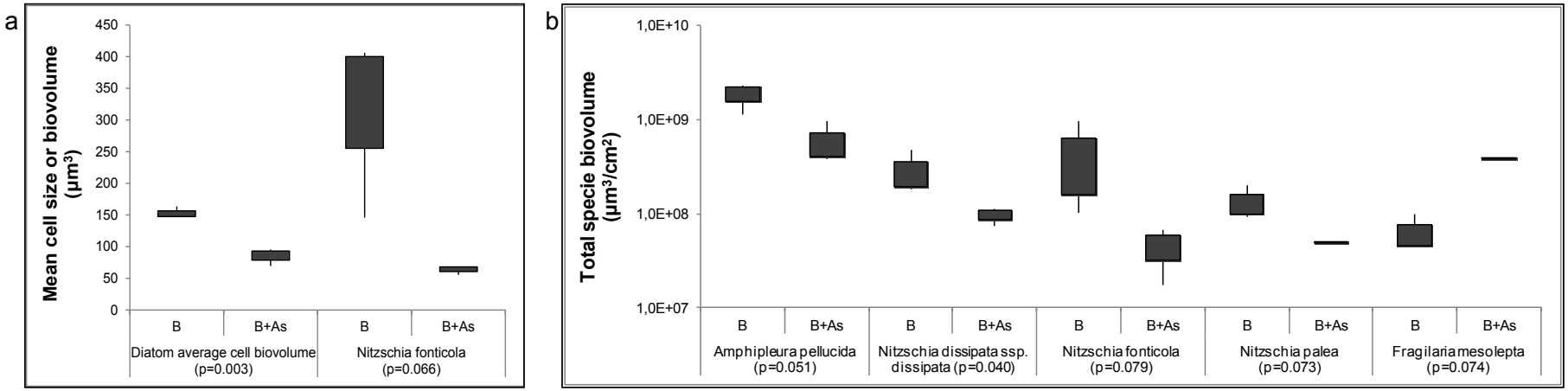


Figure 6 Boxplots representing changes in (a) average diatom cell size (μm^3) and (b) total diatom species biovolume ($\mu m^3\ cm^{-2}$), of significant and some marginal significant species. Y-axis is log transformed.

In addition, measured biovolumes were compared with theoretical biovolume data (http://hydrobio-dce.irstea.fr/cours-deau/diatomees/) for each species and were poorly correlated (R^2=0.039).

4. DISCUSSION

The arsenic concentration used in this experiment was low compared to the CMC (acute arsenic exposure) established by the US EPA (2014) in freshwater. Despite this, it affected biofilm structure and function. These effects were expected based on low measured phosphate concentrations, similar to the experiment of Rodriguez Castro *et al.* (2015). However, it does not agree with our expectation concerning the influence of fish on phosphate concentration.

After fish addition, higher dissolved phosphate concentrations were found in all treatments (about 13 µg L^{-1}), except in arsenic alone. However, these phosphate concentrations still remained limiting according to Dodds *et al.* (1998). Therefore, despite fish addition, the expected protection role of phosphate for algae was not fully achieved. Compared with Rodriguez Castro *et al.* (2015), final phosphate concentration was not high enough to protect all algae from arsenic toxicity. A possible explanation for this might be related to fish density, which was not high enough to provide enough nutrients via their excretion, and/or mineralization rates, which was not high enough to produce high phosphate concentration from organic matter (fish excretion) to overcome algal uptake.

Fish addition accelerated algal growth (Fig. 3), which corresponded to the higher phosphate concentration in water, which in turn was probably a result of fish metabolism (***Chapter 2***). Although phosphate is one of the most important determinants of algal production (Borchardt 1996), biofilm growth was delayed by arsenic exposure. Thus, it seems that arsenic prevented the uptake of phosphate by algal biofilm, as shown in Rodriguez Castro *et al.* (2015), which resulted in growth inhibition, caused also by the direct As-toxicity. The lower photosynthetic efficiency in P-limited conditions leading to lower algal growth has also been observed previously (Rodriguez Castro *et al.* 2015). During biofilm formation, algal succession usually begins with the emergence of diatoms, followed by cyanobacteria and finally filamentous green algae (Romaní 2010; Bonet 2013). However, arsenic impeded filamentous green algae growth and caused diatoms to dominate by the end of the experiment, leading to lower temporal variability (Fig. 4). Changes in *Ymax* at the end of the experiment indicated that important structural changes in photosystem II (PSII) occurred in biofilm exposed to arsenic (Fig. 5). Therefore, arsenic inhibits the potential maximum photosynthetic activity of algal biofilm in conditions of phosphate limitation, confirming the recent findings of Rodriguez Castro *et al.* (2015). In addition, the measures given by the PhytoPAM were in concordance with an increase of oxygen concentration in the water, which indicates that the main kind of photosynthesis in the system was an oxygenic photosynthesis. Therefore, arsenic caused biofilm to become less

phototrophic, what is also supported by the fact that, in contrast to algae, live bacterial cell densities did not decrease (chlorophyll-*a* concentration halved, Table 2). Thus, the proportion of biofilm consisting of algae decreased. Bacterial resistance to arsenic has already been documented (e.g. Davolos and Pietrangeli 2013). A general reduction of the *Yeff* parameter (Fig. 5) shows that arsenic caused a less efficient photosynthesis in algae (Corcoll *et al.* 2012a). However, diatoms were able to recover their photosynthetic efficiency at the end of the experiment, indicating adaptation of the diatom community to arsenic exposure.

Diatoms are cosmopolitan algae and predominate in fluvial biofilms. Diatom communities exposed to metals have variable capacities to tolerate the stress caused by the toxicant, both at the individual scale (with different levels of sensitivity among species) and at the community scale, where the biofilm acts as a coherent and protective matrix (Morin *et al.* 2012). Diatom taxonomical identification was carried out with samples taken on the last day of the experiment, when community structure was mature and expected to show the accumulated effects of 13 days of arsenic exposure. Besides the global shift in algal composition, the diatom community responded through a decrease in species richness, already documented as an effect of metal pollution (Morin *et al.* 2012). However, total diatom density remained relatively unaffected. Therefore, while total algal biomass was affected by arsenic, there was a delay in the expected replacement of diatoms by filamentous green algae due to succession, leading to similar values of diatom density at the end of the experiment. This was attributed to different processes (succession *vs.* selection pressure linked with arsenic exposure), which caused clear effects on cell size and slight changes in species composition. *Achnanthidium minutissimum*, a metal-tolerant species (see review in Morin *et al.* 2012), was the most abundant species found, representing almost the 77% of the total abundance of diatoms. *Achnantidium minutissimum* is also considered tolerant to nutrient limitation, and its small cell size is a key feature that allows maintenance of larger populations and broader regional distributions than larger species (Passy 2008). In addition, the shift towards its higher abundances in arsenic exposed communities (from 75% in the *B* treatment to almost 79% in *B+As*), highlighted its tolerance to arsenic. For other species found, 30% increased in cell numbers. In particular *Ulnaria ulna,* a species known for its resistance to metals (McFarland *et al.* 1997; Blanck *et al.* 2003; Tien 2004; Duong *et al.* 2008; Ferreira da Silva *et al.* 2009), achieved larger populations in the arsenic treatment.

In addition, arsenic clearly caused a global decrease in the average diatom cell size or cell biovolume (Table 5 and Fig. 6a), a phenomenon also observed in some individual species, such as *Nitzschia fonticola*. According to Morin *et al.* (2012), community size may be affected in several complementary ways: as a reduction of cell number, and/or a diminution of cell size within a given species. Reduction of cell size within taxa with metal exposure can be linked to the peculiar mitotic division during vegetative reproduction in diatoms, which is different to that of other algae. Each division results in two daughter cells, one of which is the same size as the mother cell, with the other being smaller. As a consequence, average cell size at the population level is reduced with each successive round of mitosis (Drebes 1977). Vegetative reproduction

is the dominant mode of multiplication in diatoms (Chepurnov *et al.* 2008), so this decrease in size could be a result of a higher cell division rate in organisms that live in stressed ecosystems (Gensemer 1995; Potapova and Snoeijs 1997). The decrease in size of many taxa in metal-contaminated environments has already been observed (Cattaneo *et al.* 1998; Cattaneo *et al.* 2004; Morin and Coste 2006; Luís *et al.* 2011). Moreover, it is known that in algae there is a positive richness-body size relationship (Passy 2012), which agrees with our results. Total diatom sample biovolume, a parameter dependant on both diatom abundances and cell size, decreased in several cases, such as *Amphipleura pellucida*, *Nitzschia dissipata* spp. *dissipata* and *Nitzschia fonticola,* and increased in others including *Fragilaria mesolepta* (Fig. 6b), highlighting the different strategies used to cope with arsenic contamination. An increase in cell volume in a diatom species, *Cylindrotheca fusiformis,* with copper exposure has also been attributed to a tolerance mechanism (Pistocchi *et al.* 1997). Summarizing, both higher *Achnanthidium minutissimum* relative abundances and greater abundance of smaller cell size diatoms were the two main changes favored under arsenic exposure. This supports the idea that large organisms are more sensitive to stress than short-lived and fast-reproducing small ones. This size-dependent sensitivity holds many implications for community functions: systems under stress would be dominated by smaller organisms with faster metabolism and flux rates. Thus, body size is a fundamental measured property of single organisms and whole communities. In addition, our results highlight the importance of taking cell biovolume real measures in water quality assessments or ecotoxicology studies based on diatoms.

The direct effects observed on biofilm function, structure and their dynamics (succession) could cause indirect effects on water chemistry. For example, a resultant increase in water conductivity may cause a decrease in the capacity of algae to take and hold solutes, which are necessary for photosynthesis; while a decrease in dissolved oxygen concentration reflects oxygen consumption by bacteria and the strong decrease in oxygenic photosynthesis activity (Table 1). A lower ability of biofilm to oxygenate the system could be therefore expected as an indirect effect of arsenic exposure.

Finally, it is necessary to highlight that this experiment with arsenic was very short (only 13 days), but still resulted in strong effects on biofilm and especially in diatoms. Furthermore, this experiment was a dynamic system with fish, making it more realistic than the classic short-term effect test with algae. Therefore, it is important to be aware that the long-term impact in a real polluted ecosystem would be different and probably much higher. In addition, the recovery would be more difficult since structural changes were also observed.

5. CONCLUSIONS

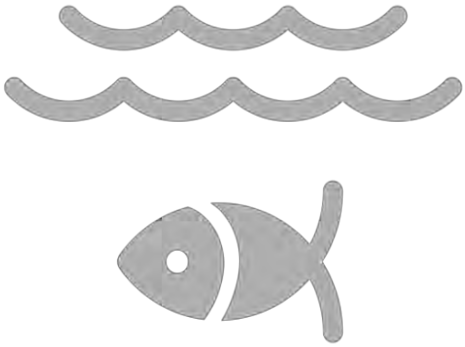
Knowing that chronic exposure of 130 µg As L^{-1} is commonly found in naturally As-enriched fluvial systems (Rosso *et al.* 2011), we conclude and highlight that short-term biofilm exposure to arsenic at environmentally realistic concentrations (130 µg L^{-1} during 13 days) under P_limited conditions, was sufficient to cause direct effects on algae. Using chlorophyll-a as a measure of algal biomass, and live bacteria number as an approximation of bacterial biomass, we conclude that a less phototrophic biofilm was developed, as algal growth and productivity were reduced. Moreover, arsenic impeded the algal succession process, causing changes in the algal community and specifically in diatoms: a loss of diatom species sensitive to arsenic and a significant decrease in cell size may allow diatoms to become more tolerant to the toxicant. Therefore, an important function of the system was lost, regarding to the decrease of primary production and the loss of biodiversity. All these changes have obvious ecological implications for freshwater environments, especially rivers. Considering how low arsenic concentration and exposure time were in this experiment compared with reality, the results call into question the limits of arsenic concentration established by the US EPA (2014) in freshwater based on acute arsenic exposure (340 µg L^{-1}).

The protection role of phosphate for algae exposed to arsenic was not fully achieved. Further experiments are needed to disentangle and better understand the complex set of processes contributing to arsenic and phosphate cycling by decomposers, primary producers and consumers.

Finally, we strongly support the use of biofilm and a multi-endpoint approach to measure effects of toxicants in freshwater ecosystems. This study also brings new arguments for the use of real measurements in the estimation of diatom biovolume (cell size), as well as for the use of multi-trophic studies to elucidate the real effects of toxicants.

CHAPTER 2

BEHAVIORAL AND PHYSICAL EFFECTS OF ARSENIC EXPOSURE IN FISH ARE AGGRAVATED BY AQUATIC ALGAE



Magellan K, Barral-Fraga L, Rovira M, Srean P, Urrea G, García-Berthou E, Guasch H. (2014). Behavioural and physical effects of arsenic exposure in fish are aggravated by aquatic algae. *Aquatic Toxicology,* 156:116-124.

doi: http://dx.doi.org/10.1016/j.aquatox.2014.08.006 (see Annex 2)

ABSTRACT

Arsenic toxicity on freshwaters depends on numerous interacting factors which makes effects difficult to estimate. The use of aquatic algae is often advocated for bioremediation of arsenic contaminated waters as they absorb arsenate and transform it into arsenite and methylated chemical species. Fish are another key constituent of aquatic ecosystems. Contamination in natural systems is often too low to cause mortality but sufficient to interfere with normal functioning. Alteration of complex, naturally occurring fish behaviors such as foraging and aggression are ecologically relevant indicators of toxicity and ideal for assessing sublethal impacts. We examined the effects of arsenic exposure in the invasive mosquitofish, *Gambusia holbrooki*, in a laboratory experiment incorporating some of the complexity of natural systems by including the interacting effects of aquatic algae. Our aims were to quantify the effects of arsenic on some complex behaviors and physical parameters in mosquitofish, and to assess whether the detoxifying mechanisms of algae would ameliorate any effects of arsenic exposure. Aggression increased significantly with arsenic whereas operculum movement decreased non-significantly and neither food capture efficiency nor consumption was notably affected. Bioaccumulation increased with arsenic and unexpectedly so did fish biomass. Possibly increased aggression facilitated food resource defense allowing fish to gain weight. The presence of algae aggravated the effects of arsenic exposure. For increase in fish biomass, algae acted antagonistically with arsenic, resulting in a disadvantageous reduction in weight gained. For bioaccumulation the effects were even more severe, as algae operated additively with arsenic to increase arsenic uptake and/or assimilation. Aggression was also highest in the presence of both algae and arsenic. Bioremediation of arsenic contaminated waters using aquatic algae should therefore be carried out with consideration of entire ecosystem effects. We highlight that multidisciplinary, cross-taxon research, particularly integrating behavioral and other effects, is crucial for understanding the impacts of arsenic toxicity and thus restoration of aquatic ecosystems.

1. BACKGROUND

Arsenic (As) from both anthropogenic and natural sources has global impacts (Mandal and Suzuki 2002; Nordstrom 2002; Rahman and Hasegawa 2012; Rahman *et al.* 2012; Smedley and Kinniburgh 2002) and aquatic systems, including freshwaters, are major repositories for arsenic (Nordstrom 2002; Smedley and Kinniburgh 2002). Although some national and international standards are in effect, for example the World Health Organization safe limit for drinking water is 10 μg L^{-1} (Smith *et al.* 2002), the toxicity of arsenic is dependent on numerous interacting factors such as its source, concentration and bioavailability; environmental parameters; and organisms' resistance ability and detoxifying mechanisms (Mandal and Suzuki 2002; Rahman and Hasegawa 2012; Smedley and Kinniburgh 2002). A key factor is its chemical speciation. Inorganic arsenic (iAs) is generally more toxic than organic As,

while of the iAs species, arsenite (As^{III}) is more toxic than arsenate (As^{V}). However, the organic methylated species (dimethylarsenite, DMA^{III}, and monomethylarsenite, MMA^{III}) are more toxic than their iAs parent compounds (Rahman *et al.* 2012; Smedley and Kinniburgh 2002). Quantifying total arsenic in environmental and biological samples is therefore not synonymous with assessment of associated risks. The main chemical species in freshwaters are inorganic arsenics but methylated and other organic arsenic species are also found (Rahman and Hasegawa 2012; Rahman *et al.* 2012). Freshwater ecosystems are extensive and highly dynamic (Moss 1998) which together with the variable nature of arsenic toxicity makes effects difficult to estimate (Rahman *et al.* 2012; Smedley and Kinniburgh 2002; Smith *et al.* 2002). However, assessment and prediction are essential. In addition to providing water and nutrients for human consumption (Mandal and Suzuki 2002; Smith *et al.* 2002; Villéger *et al.* 2012), freshwater ecosystems may themselves suffer severe impacts from arsenic toxicity (e.g. Rahman and Hasegawa 2012; Rahman *et al.* 2012; Scott and Sloman 2004; Smedley and Kinniburgh 2002).

Biological activity plays a vital role in arsenic speciation, distribution and cycling in freshwaters (Rahman and Hasegawa 2012; Rahman *et al.* 2012). Organismal uptake of arsenic may be direct, through ingestion, inhalation and absorption, or indirect through the food chain (Mandal and Suzuki 2002; Moss 1998; Smedley and Kinniburgh 2002; Smith *et al.* 2002). Microalgae (and bacteria) have important functions in these processes through biotransformation of arsenic species (Hellweger and Lall 2004; Rahman and Hasegawa 2012; Rahman *et al.* 2012). Algae mistake As^{V} for PO_4^{3-} and actively absorb it via the same pathways. Once inside the algal cells, As^{V} becomes toxic and algae can reduce it to As^{III}, methylate it and excrete it, which is thought to be a detoxifying mechanism (Hellweger and Lall 2004; Rahman and Hasegawa 2012; Rahman *et al.* 2012). Several factors influence this process. Different algal species have different methylation abilities (Rahman and Hasegawa 2012) and tolerances to As^{V} (e.g. Favas *et al.* 2012; Levy *et al.* 2005; Wang *et al.* 2013), and not all algae excrete As^{III}. For example, both *Chlorella* sp. and *Monoraphidium arcuatum* take up As^{V} and reduce it to As^{III} but only *M. arcuatum* excretes it (Levy *et al.* 2005). Moreover, recent studies indicate that methylation may not be the primary mode of detoxification in freshwater algae. Instead, arsenic is taken up by cells using the phosphate transport system, reduced to As^{III} in the cell and then excreted into the growth medium, probably by an active transport system (Levy *et al.* 2005; Wang *et al.* 2013). For example, after exposing *Chlamydomonas reinhardtii* and *Scenedesmus obliquus* to different arsenate concentrations, no methylated species could be detected (Wang *et al.* 2013). Similarly, arsenate and arsenite were the dominant species in the freshwater algae *Synechocystis sp.* and *C. reinhardtii* (Yin *et al.* 2011, 2012). This transformation reaction is suggested to be correlated with algal growth rate and P nutrient status, leading to almost complete methylation under P-limiting conditions and slower methylation and excretion of As^{III} into the media if P is in excess (Hellweger and Lall 2004). Nonetheless, these studies confirm that P has a key role in arsenate toxicity and that biotransformation of arsenic by algae is a central component of aquatic arsenic cycling. Indeed, the use of algae is often advocated for

bioremediation of arsenic contaminated water (e.g. Levy *et al.* 2005; Favas *et al.* 2012; Rahman and Hasegawa 2012; Rahman *et al.* 2012; Wang *et al.* 2013).

Fish are a key constituent of aquatic ecosystems and are involved in arsenic mobilization. They are an important component of the aquatic food chain (Agah *et al.* 2009; Kumar and Banerjee 2012; Zhang *et al.* 2013) and even small fish are a source of protein for human consumption (e.g. Moeller *et al.* 2003). Some fish are also used as bioindicators of various aquatic pollutants (Bhattacharya *et al.* 2007; Moeller *et al.* 2003; Moss 1998; Scott and Sloman 2004). Bioaccumulation of arsenic in fish occurs directly through absorption across the gills or skin and indirectly via consumption of prey (Rahman *et al.* 2012); and inorganic, methylated and other organic arsenicals are all found in various fish species (Rahman *et al.* 2012; Rahman and Hasegawa 2012). The effects of arsenic toxicity have been examined in numerous species worldwide. For example, bioaccumulation of arsenic has been recorded in fish from California (Moeller *et al.* 2003), sub-Saharan Africa (Ouédraogo and Amyot 2013), India (Kumar and Banerjee 2012), France (Noël *et al.* 2013), China (Zhang *et al.* 2013) and the Persian Gulf (Agah *et al.* 2009). However, most research has focused on parameters such as bioaccumulation, and physiological parameters such as growth (e.g. Kumar and Banerjee 2012) and metabolic and histopathological effects (e.g. Ahmed *et al.* 2013; Bhattacharya *et al.* 2007). One factor that has received much less attention is fish behavior (e.g. Scott and Sloman 2004; Weis and Candelmo 2012; Weis *et al.* 2001). Contamination in natural systems is often at concentrations well below those that cause mortality, but even low levels of toxicity may be sufficient to interfere with normal functioning. Fish behavior is ideal for assessing these sublethal impacts (Moss 1998; Scott and Sloman 2004; Weis and Candelmo 2012). Much of the current research focusses on direct behavioral responses to contaminants, for example, avoidance of contaminated sites, respiratory changes and behavior like body tremors associated with illness. However, alteration of complex, naturally occurring behaviors such as foraging and predation, agonistic interactions, shoaling and reproductive behaviors are more ecologically relevant indicators of toxicity (Scott and Sloman 2004; Sopinka *et al.* 2010; Weis *et al.* 2001). Various environmental toxicants have been shown to affect complex behaviors (reviewed in Atchison *et al.* 1987; Scott and Sloman 2004). Arsenic in particular reduces long-term memory in the zebrafish, *Danio rerio* (de Castro *et al.* 2009) and is part of a cocktail of chemicals that affects aggressive interactions in the round goby, *Neogobius melanostomus* (Sopinka *et al.* 2010). However, the effects of arsenic on fish behavior have received little attention to date: arsenic is not listed in Scott and Sloman's (2004) comprehensive review of contaminant effects on fish behavior. Given the global impacts of arsenic toxicity (e.g. Mandal and Suzuki 2002; Smedley and Kinniburgh 2002; Rahman *et al.* 2012) more work is needed in this field.

In this study, we examined the effects of arsenic on complex behaviors in the invasive mosquitofish, *Gambusia holbrooki*. This small fish has been introduced worldwide, primarily for mosquito control (Lever 1996; Pyke 2008). Although highly tolerant of a variety of stressors (e.g.

Evans *et al.* 2011; Staub *et al.* 2004; Uliano *et al.* 2010), *G. holbrooki* and the closely related *Gambusia affinis* have been used in toxicity studies (e.g. Tatara *et al.* 1999, 2001) and are known to be affected by arsenic (e.g. Moeller *et al.* 2003; Newman *et al.* 1989). Since behavior links physiological functions with ecological processes, an understudied field of research (e.g. Scott and Sloman 2004; Weis *et al.* 2001), we also included physiological parameters to assess interrelated effects of arsenic toxicity. Moreover, given the intricacies of the feedback and cycling interactions contributing to arsenic toxicity (e.g. Scott and Sloman 2004; Weis *et al.* 2011), field studies may be more general and realistic about environmental effects (Moss 1998), while laboratory studies allow more controlled quantification of effects, and both provide valuable insight (Weis and Candelmo 2012). Therefore, we also examined the interacting effects of naturally occurring algae, thus incorporating some of the complexity of natural systems in a laboratory experiment and disentangling some specific processes from whole ecosystem effects.

We addressed two main aims: first to quantify the effects of arsenic on *G. holbrooki*, and second to assess the interacting affects of algae on arsenic toxicity in this fish species. We examined one direct behavioral response to stress, opercular ventilation rate (Brown *et al.* 2005; Hawkins *et al.* 2004), predicting that operculum movement would increase in response to the stress of arsenic exposure; and two complex behaviors, aggression and foraging. Since both stress (Folkedal *et al.* 2012) and physiological effects of contaminants (Weis *et al.* 2001) can reduce feeding ability and motivation, we predicted that food capture and consumption would be decreased with arsenic exposure. For aggression the effects of toxicant exposure are more ambiguous, provoking both increases and decreases in aggression (Scott and Sloman 2004; Sopinka *et al.* 2010) so while we expected to see a difference with arsenic exposure we made no directional predictions. Then, for physical parameters, we predicted that fish would gain less weight (e.g. Kumar and Banerjee 2012) but increase bioaccumulation (Scott and Sloman 2004) in the presence of arsenic. Finally, given the various and interrelated influences on algal arsenic detoxification capacity we hypothesized that freshwater algal communities will affect As^{V} toxicity to fish, but the direction of effects is, *a priori*, difficult to predict.

2. METHODS

2. 1. Experiment

Mosquitofish were collected from the Ter (42.0451º N, 3.1960º E), Fluvià (42.1875º N, 3.0851º E) and Muga (42.2527º N, 3.0756º E) rivers and transported to the laboratory where they were placed in 60 L stock aquaria (60 cm × 30 cm × 32 cm) each containing a gravel substrate, conditioned water and a filtered air supply. *G. holbrooki* from all three rivers were housed together. Aquaria were maintained at 25 ± 1 ºC and a constant photoperiod (12:12 h light:dark cycle) using 6W bulbs. Fish were fed to satiation once per day with commercial food

flakes or frozen bloodworms (*Chironomus* spp.) and were able to acclimate to laboratory conditions for at least 6 months, with a further month to acclimate to experiment-specific environmental parameters (e.g. temperature: see below).

For the experiment, 12 independent sets of apparatus (experimental units) were set up (see Fig. 1 on ***Chapter 1***). A large (sump) tank (60 cm × 25 cm × 75 cm) was filled with 90 L of filtered water. A smaller (fish) tank (31.5 cm × 11 cm × 31.5 cm) containing 4 L of filtered water was placed on top, and above this was placed a channel (90 cm × 8.5 cm × 7.5 cm) containing sandblasted glass tiles (1 cm^2) to provide substrate for the algal biofilm. 10 g L^{-1} each of phosphate and silicate were added once per week to reproduce phosphate limiting conditions for algal growth, i. e. stationary growth phase (Hellweger and Lall 2004; Moss 1998; Rahman and Hasegawa 2012), and to facilitate diatom growth respectively. Water was pumped from the large tank to the head of the algal biofilm channel, passed through this channel into the fish tank, circulated in the fish tank then passed through the overflow back into the sump tank (see Fig. 1 on ***Chapter 1***). The overflow was covered with a fine mesh to prevent algae and fish from leaving via this route. Water levels were monitored throughout the experiment. Water pH was maintained at 7. 5 using a pH control system based on CO_2 addition (JBL Proflora m630: JBL, Ludwigshafen, Germany) to provide enough inorganic carbon for algal growth (Favas *et al.* 2012; Smedley and Kinniburgh 2002). Illumination (12 h light:12 h dark) was provided by 120W LED Grow Lights (Lightech, Girona, Spain) which produce light without heat, and temperature was maintained at 19.5 ± 5 ºC. This is quite a low temperature for mosquitofish, but well within their tolerance range (Evans *et al.* 2011), and was necessary for algal growth. The experimental units were left to condition for 1 week prior to the start of the experiment.

Natural algal inocula were obtained from the Llémena stream, a tributary of the Ter River, by scraping three cobbles from the upstream zone which has minimum human impact (see Serra *et al.* 2009). On day 1 of the experiment, and at weekly intervals during the following 19 days, the inocula were added to the channels of half of the experimental units so that biofilm was able to colonize the glass tiles. On day 20, 130 g L^{-1} of arsenate was added to the sumps of half of the experimental units. Arsenate was used as this is the most common arsenic species in freshwater and is the species that is taken up by aquatic algae (Hellweger and Lall 2004; Rahman and Hasegawa 2012; Rahman *et al.* 2012). This gave 3 replicates each of 4 conditions: control (C) with neither As^V nor biofilm, biofilm (B), arsenic (A) and biofilm with arsenic (B + A). On day 24, all fish were weighed to the nearest mg using a balance and total length (TL) was measured to the nearest mm using a ruler. Four fish were added to each experimental unit: 1 male (26.8 ± 2.89 mm TL; mean ± standard deviation) and 3 females (1 small: 28.6 ± 5.51 mm TL; 1 medium: 39.4 ± 1.78 mm TL; 1 large: 45.3 ± 2.96 mm TL). This sex ratio was chosen to reduce sexual harassment of females by males (Evans *et al.* 2011; Meffe and Snelson 1989) and as fish numbers were limited. Different sized females were used primarily to allow identification of individuals within a tank so any overlap in sizes between tanks was tolerated. Video recorded observations began on day 25 and continued for 9 days during

which arsenic was measured every day and phosphate was measured every 3 days (Table 1). The video camera was placed approximately 50 cm in front of the narrow sides of the fish tanks. Pilot observations showed that fish were not disturbed by the camera. Each day one 10-min video was taken of each tank. Immediately following this, five defrosted frozen bloodworms were added sequentially to each tank such that one prey was consumed before the next was added (also videoed). The order in which tanks were videoed was randomized daily. After observations, all fish were fed to satiation. Any excess food was removed after 1 h and fish were left until the following day. Any fish that died during the experiment (n = 4) were replaced immediately with a same sex, similar sized individual. This occurred only in the first three days of experiments and in all cases except one were males.

Table 1 Total arsenic and phosphate concentrations ($\mu g\ L^{-1}$: mean ± standard deviation) during the 9 days of observations. For As: n = 9 and P: n = 3.

Treatment	Arsenic	Phosphate
Control	1.92 ± 0.09	12.11 ± 4.10
Biofilm (B)	1.89 ± 0.11	12.28 ± 3.34
Arsenic (A)	127.96 ± 5.55	3.18 ± 1.17
B + A	124.20 ± 2.64	15.96 ± 4.14

After the final observations, all fish were euthanized using an overdose of anesthetic (clove oil) and weighed and measured as before. Liver and gills were dissected out of each female for analysis of tissue arsenic accumulation. These organs were selected as both are crucial sites of metabolic activity so are likely to accumulate arsenic (e.g. Ahmed *et al.* 2013; Kumar and Banerjee 2012). Only females were used for this analysis to avoid biases due to sex differences in bioaccumulation, and as it requires a minimum amount of tissue the single male in each tank was unlikely to be sufficient. To quantify the total amount of arsenic accumulated in fish, the dissected samples were frozen, then freeze-dried, then digested with 4 ml of concentrated HNO_3 (65% HNO_3, Suprapur, Merck, Germany) and 1 ml of H_2O_2 (31% H_2O_2, Suprapur, Merck, Germany). Next, a 75-times dilution with milliQ water and acidification (1%) of the samples was performed. Digested samples were analyzed following the procedure used for total arsenic in water. Bioaccumulation was expressed as dissolved arsenic per dry weight (g arsenic g DW^{-1}). Total dissolved arsenic concentration was measured by ICP-MS (7500c Agilent Technologies, Inc., Wilmington, DE). The detection limit for arsenic was 0.08 g L^{-1}. Rh was used as the internal standard. The accuracy of the analytical method was checked

periodically using certified water reference (SPS-SW2 Batch 113, Oslo, Norway).

This work followed all national and institutional guidelines for animal experiments and every effort was made to ensure that suffering to the fish was minimized.

2. 2. Video and statistical analyses

Direct behavior

The frequencies of opercular movements were recorded for each individual by counting the number of times the operculum opened. Since opercula were not always visible, this variable was recorded for a total of approximately 1 min and converted to opercula beats per minute for analyses. In a few cases the fish remained hidden throughout the observation for that day so these observations were excluded from analyses. To assess differences in aggression between treatments, opercula beats min^{-1} were used in a generalized estimating equation (GEE: an extension of generalized linear models developed for situations where response variables are correlated rather than independent). Experimental unit was the between subjects factor and time (day) was the within subjects factor for the model. The fully factorial analysis included two independent factors, presence and absence of biofilm and arsenic, and time was included as a covariate.

Complex behaviors

We recorded the frequencies of aggressive interactions initiated for each fish. These included lunges (rapid movement towards another fish without physical contact), chases (prolonged movement towards another fish with the recipient individual swimming away from the attacker), and bites (as lunges but with physical contact). Since the largest female initiated almost all aggressive interactions in all tanks only these data were used for analyses. We then used the same model as above with number of attacks carried out by the largest female as the dependent variable. Two foraging parameters were obtained: the time required to locate and capture each food item (capture efficiency), quantified as the interval between the food item touching the surface of the water and the first fish grasping the food; and the interval between capture and when each food item was fully consumed (consumption). The means of each of these variables in each tank for each day were calculated and used in separate GEEs as above.

Physical parameters

We also recorded two physical parameters. First, the change in biomass was obtained by subtracting the weight of each fish at the beginning of the experiment from its weight at the end. Any fish that had replaced a deceased individual were excluded from this analysis. These data were used as the dependent variable in a GEE with experimental unit as the between

subjects variable and fish number within each tank as the within subjects variable. The final, factorial model included presence and absence of biofilm and arsenic as independent factors and total length of each fish as a covariate. Second, the tissue concentration of arsenic for the females in each tank was the dependent variable in a factorial generalized linear model (GLM) with the presence and absence of biofilm and arsenic, and the summed changes in biomass for all females in each tank (obtained from the previous analysis) as independent factors. Analyses were conducted using SPSS v. 20. All dependent variables were analyzed with normal distributions and identity link functions.

3. RESULTS

3. 1. Direct behavior

Operculum movement was highest in the control and lowest with just biofilm present. Arsenic produced a lesser decrease in operculum movement whether or not biofilm was present (Fig. 2). Opercular movements increased significantly over time (Table 2, Fig. 2) and there was a significant interaction between time and all other variables while the presence of biofilm and arsenic and their interaction were non-significant (Table 2).

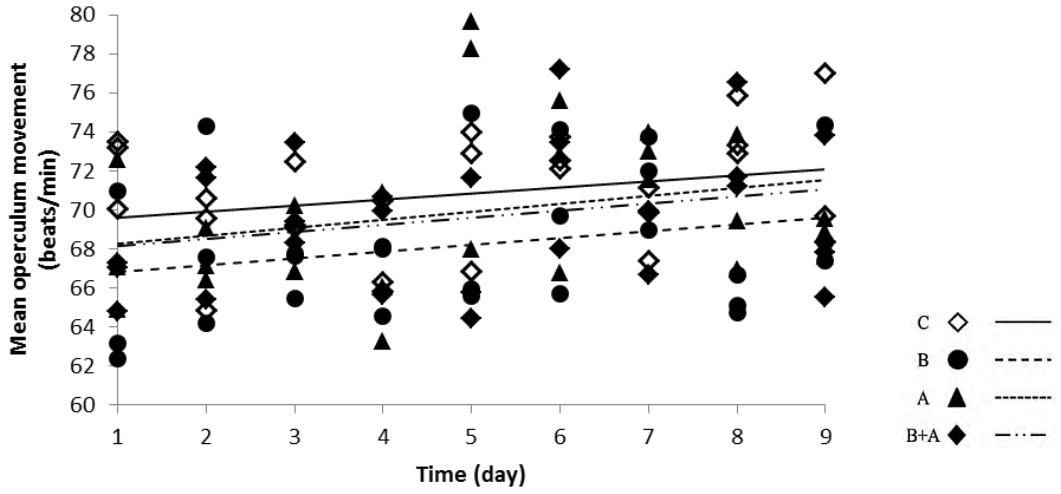


Figure 2 Mean opercular movements for all four fish in each tank. Trend lines illustrate the relationships between time and the presence and absence of biofilm and arsenic. C = control; B = biofilm; A = arsenic; B+A = biofilm + arsenic.

Table 2 Results for the generalized estimating equations for variations in operculum movement (beats min^{-1}) and aggression. Significant results are highlighted.

Variable	Operculum Movement			Aggression		
	Wald χ^2	*df*	*p*	Wald χ^2	*df*	*p*
Biofilm (B)	2.977	1	0.084	5.061	1	**0.024**
Arsenic (A)	0.025	1	0.876	11.898	1	**0.001**
Time (T)	110.179	8	**<0.001**	76.810	8	**<0.001**
B × A	2.121	1	0.145	1.102	1	0.294
B × T	242.592	8	**<0.001**	13.652	8	0.091
A × T	40.374	8	**<0.001**	18.053	8	**0.021**
B × A × T	207.470	8	**<0.001**	3.910	8	0.865

3. 2. Complex behaviors

Aggression was lowest in the control. Although biofilm presence initially induced an increase in aggression, this appeared to be returning to the same level as the controls (Fig. 3). Aggression increased almost linearly in the presence of arsenic, and was highest in the presence of both arsenic and biofilm (Fig. 3). The frequency of aggression increased significantly with all three independent factors (Table 2, Fig. 3); however, while the interaction between time and arsenic presence was significant, that between time and biofilm presence was marginally non-significant (Table 2). All other interactions were non-significant (Table 2).

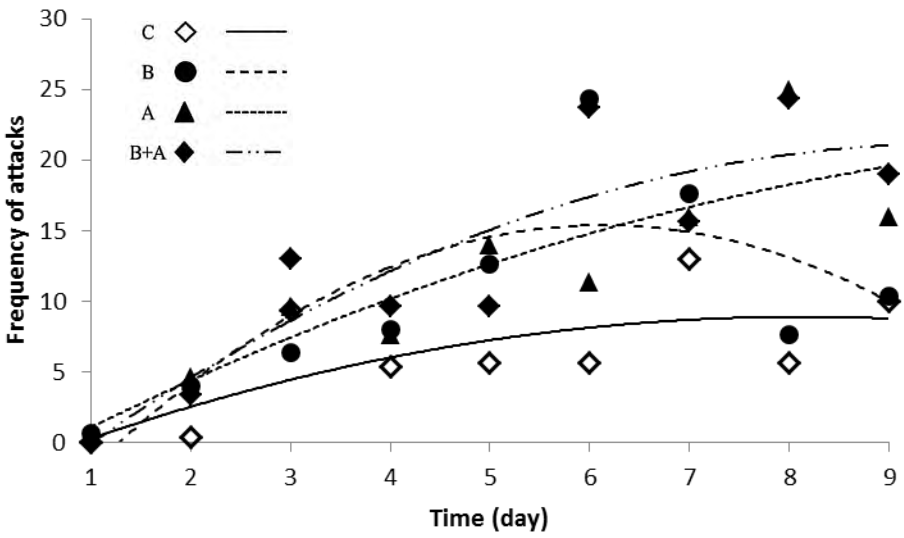


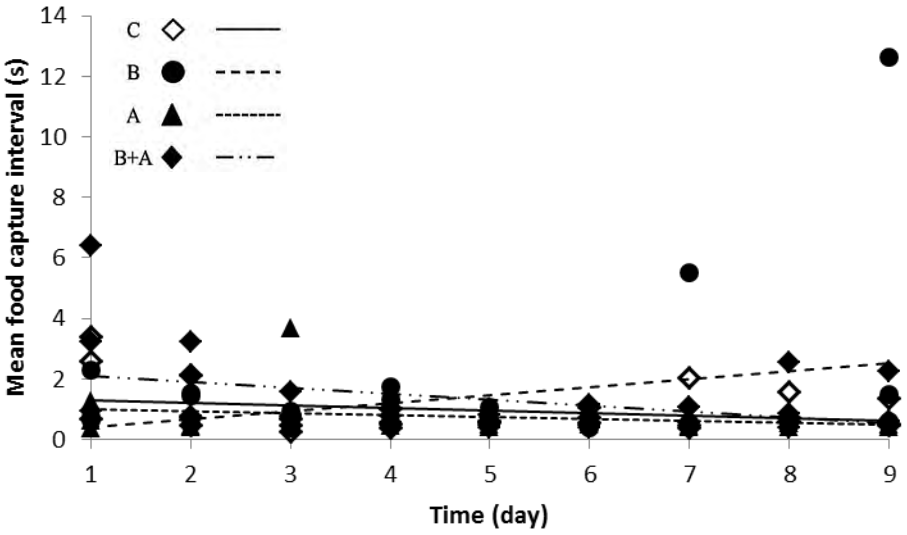
Figure 3 The frequency of attacks carried out by the largest female in each tank on each day. Best fit (quadratic) trendlines have been added to illustrate the relationships between time and the presence and absence of biofilm and arsenic. C = control (r^2 = 0.53); B = biofilm (r^2 = 0.63); A = arsenic (r^2 = 0.80); B+A = biofilm + arsenic (r^2 = 0.78).

Time had the greatest effect on both foraging variables with capture interval generally significantly decreasing and consumption interval generally significantly increasing over time (Table 3, Fig. 4). However, capture interval increased significantly in the presence of biofilm (Table 3, Fig. 4a), though this may be an artefact resulting from unusually high values in one tank towards the end of the experiment which may have been caused by external disturbance. We retained this outlier in analyses to maintain sample size. The interaction between time, biofilm and arsenic presence was also significant while all other variables and their interactions were non-significant (Table 3). For food consumption interval the only other significant interaction was between the presence of biofilm and the presence of arsenic (Table 3), though again this may reflect the later high values for biofilm presence in one tank (Fig. 4b).

Table 3 Results for the generalized estimating equations for variation in foraging parameters. Significant results are highlighted in bold.

Variable	Capture			Consumption		
	Wald χ^2	*df*	*p*	Wald χ^2	*df*	*p*
Biofilm (B)	5.816	1	**0.016**	2.759	1	0.097
Arsenic (A)	0.601	1	0.438	1.075	1	0.300
Time (T)	25.578	8	**0.001**	51.362	8	**<0.001**
B × A	0.013	1	0.909	6.611	1	**0.010**
B × T	10.303	8	0.244	7.205	8	0.515
A × T	8.315	8	0.403	6.690	8	0.570
B × A × T	20.873	8	**0.007**	13.325	8	0.101

a)



b)

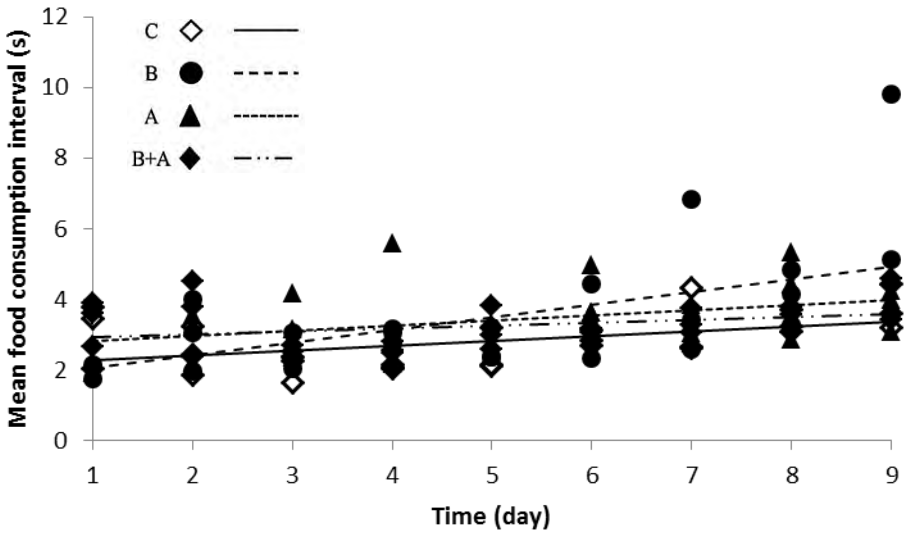


Figure 4. The mean time taken to a) capture and b) consume all five food items in each tank each day. Trendlines have been added to illustrate the relationships between time and the presence and absence of biofilm and arsenic. C = control; B = biofilm; A = arsenic; B+A = biofilm + arsenic.

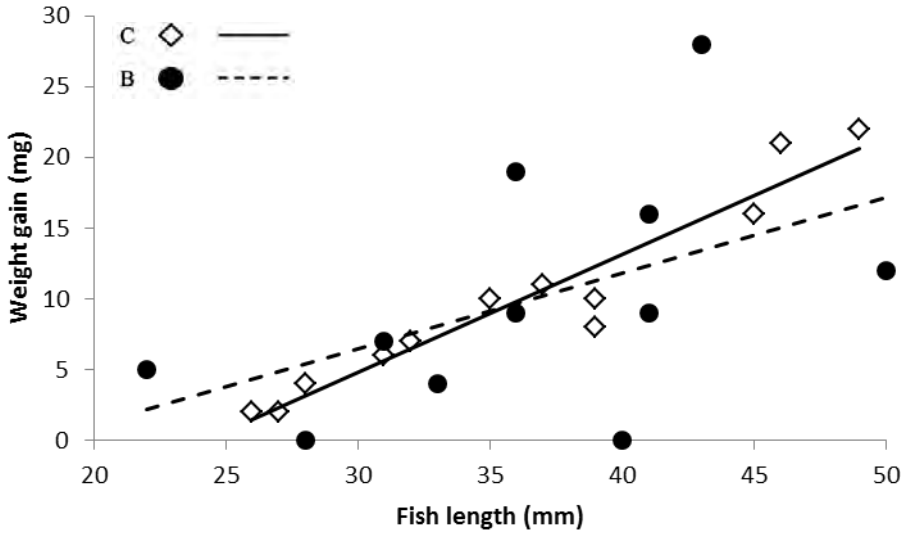
3.3 Physical parameters

All fish gained weight during the experiment (Fig. 5) and there was a significant positive relationship between weight gain and fish length (Table 4, Fig. 5). Biofilm alone showed no effect on weight gain (Table 4) though there was a significant interaction between these two variables (Table 4, Fig. 5a). However, the relationship is unclear. While weight gain increased with fish length, biofilm appears to affect smaller fish more than larger ones and the data is a widely scattered (Fig. 5a). Arsenic had a significant effect on weight gain and showed a significant interaction with both length and biofilm presence and the three-way interaction was likewise significant (Table 4). However, somewhat surprisingly weight gain increased in the presence of arsenic (Fig. 5b) and while the presence of biofilm to some extent appears to ameliorate this effect this is more apparent for smaller than larger fish (Fig. 5c).

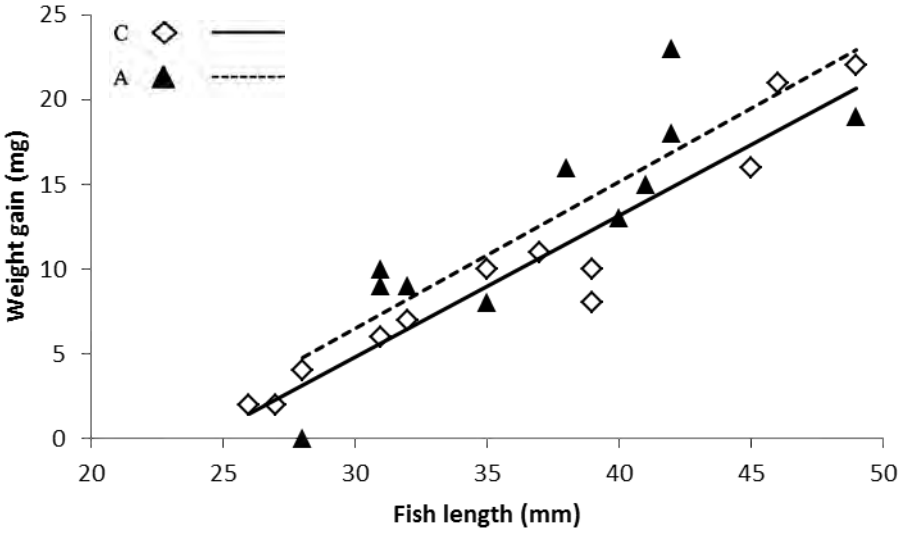
Table 4 Results for the generalized estimating equations for variations in physiological parameters. Significant results are highlighted in bold.

Change in Biomass				**Bioaccumulation**			
Variable	**Wald χ^2**	***df***	***p***	**Variable**	**Wald χ^2**	***df***	***p***
Biofilm (B)	13.208	1	0.349	**Biofilm (B)**	4.181	1	**0.041**
Arsenic (A)	0.876	1	**<0.001**	**Arsenic (A)**	5.138	1	**0.023**
Length (L)	639.187	1	**<0.001**	**Weight (W)**	6.490	1	**0.011**
B × A	15.094	1	**<0.001**	**B × A**	4.492	1	**0.034**
B × L	18.006	1	0.051	**B × W**	4.513	1	**0.034**
A × L	3.792	1	**<0.001**	**A × W**	7.784	1	**0.005**
B × A × L	13.494	1	**<0.001**	**B × A × W**	3.253	1	0.071

a)



b)



c)

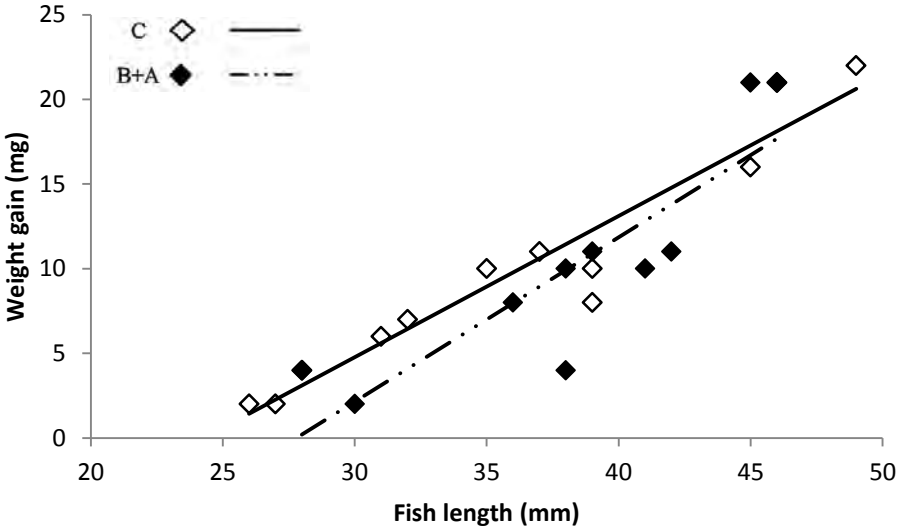


Figure 5 The change in weight between the start and end of the experiment for all fish. For clarity, each of the treatments is shown separately in comparison to the control: a) biofilm; b) arsenic; c) biofilm and arsenic. Trendlines have been added for illustration. C = control; B = biofilm; A = arsenic; B+A = biofilm + arsenic.

For tissue arsenic bioaccumulation, all factors and their interactions were significant with the exception of the three-way interaction which showed just marginal significance (Table 4). Not surprisingly, bioaccumulation was higher when arsenic was added to the water and this increased with fish weight increase (Fig. 6). Biofilm presence alone decreased arsenic bioaccumulation, presumably by removing any naturally occurring arsenic in the water. However, when biofilm and arsenic were present together, tissue arsenic accumulation showed a dramatic increase, even above that shown with arsenic alone (Fig. 6).

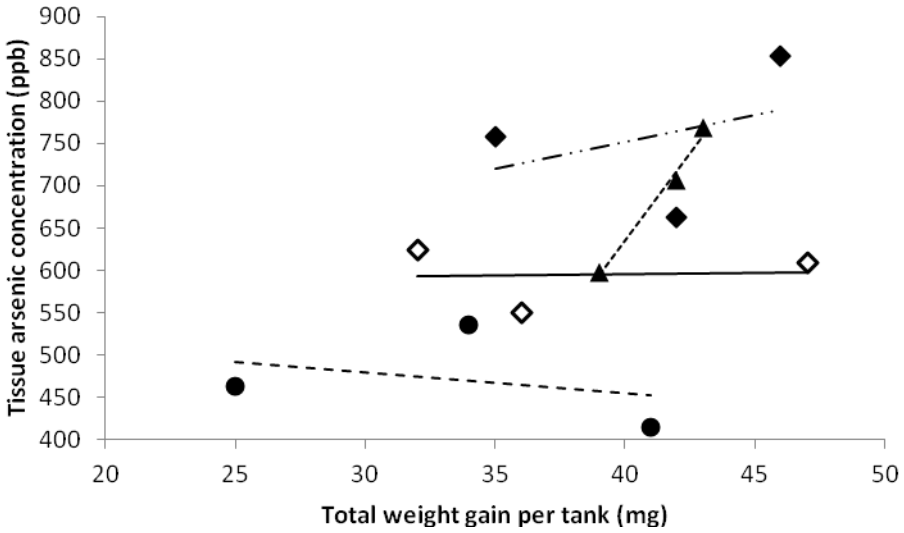


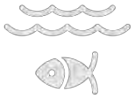
Figure 6 The differences in tissue arsenic concentration as a function of total weight gained in each tank and the presence and absence of biofilm and arsenic. Trendlines have been added to illustrate these relationships. C = control; B = biofilm; A = arsenic; B+A = biofilm + arsenic.

4. DISCUSSION

Arsenic produced some effects in mosquitofish, though not exactly as predicted. Aggression increased significantly in the presence of arsenic while for operculum movement and food capture efficiency and consumption rate time, rather than arsenic presence, was the major predictor. Aggression appears to be the major initial behavioral effect of arsenic exposure in this species and continued to increase with exposure duration. Of the behaviors measured, aggression may thus be a suitable biomarker for arsenic toxicity in mosquitofish (Moss 1998; Scott and Sloman 2004; Weis *et al.* 2001). Increased aggression may be induced through stress or related physiological changes due to arsenic exposure (e.g. Scott and Sloman 2004), which may increase the metabolic costs for an individual, thereby leading to increased stress and a potentially damaging feedback cycle. Aggression in some fish species increases with other toxicants. For example, bluegills, *Lepomis macrochirus*, exposed to copper for 96 h increased the frequency of agonistic acts (Henry and Atchison 1986), while round gobies from

contaminated sites increased their rate of assessment displays compared to fish from a reference site (Sopinka *et al.* 2010). In both these cases, dominance status played a role with more dominant bluegills increasing aggression over subordinates (Henry and Atchison 1986) and reduced dominance establishment in contaminant site gobies (Sopinka *et al.* 2010). In the present study, almost all agonistic acts were initiated by the largest, presumably dominant, female which may explain the lack of notable effects on foraging parameters. One of the major functions of aggression is resource defense, mainly defense of mates, shelter or food (Huntingford and Turner 1987; Magellan and Kaiser 2010). If the largest female was monopolizing most of the food resources, competition for the remaining food by the other individuals may mask any effects of arsenic exposure. However, foraging efficiency was only recorded for the first few food items, after which fish were fed to excess, so later effects may have been overlooked. Time had the greatest effect on foraging, the faster capture efficiency probably being due to fish learning to anticipate food and the slower consumption rate reflecting reduced motivation to feed as they gained weight. However, other factors cannot be ruled out. The concomitant increase in operculum rate over time suggests variation in oxygen demand or efficiency of oxygen uptake which may be induced by the build-up of other chemicals, such as nitrogen, naturally excreted by fish.

These behavioral results can be integrated with the physical results. All fish gained weight during the nine days of observations, probably because the few fish per tank were fed to excess each day so were released from the competition they would have experienced in the stock aquaria, which reflects the foraging results above. Larger fish gained the most weight in all treatments, although unexpectedly arsenic promoted weight gain. The reasons for this result are unknown. The accepted view is that contaminant load should cause a loss of condition (e.g. Kumar and Banerjee 2012; Scott and Sloman 2004; Weis *et al.* 2011). Increased size has been shown in grass shrimps, *Palaemonetes pugio*, from contaminated sites but this is explained by reduced predation from fish at these locations (Weis *et al.* 2011). In this study, predation was not a factor although it is interesting that weight gain and aggression varied in parallel, which may imply some effect of resource defense. Increase in fish biomass and bioaccumulation also showed similar patterns, the obvious explanation being that greater weight gain allows more arsenic to be assimilated and fixed in tissues. However, it may also be that fish that gain more weight have characteristics, such as increased aggression and therefore resource holding potential (e.g. Magellan and Kaiser 2010), that also contribute to arsenic bioaccumulation. Although we provided daily uncontaminated food, mosquitofish also consume algae and diatoms (García-Berthou 1999). The algae present in the biofilm treatments, some of which dropped into the fish part of the experimental units, were likely to be heavily contaminated with arsenic, which may have promoted bioaccumulation. Finally, small fish such as these mosquitofish, which have a large surface area to volume ratio, are particularly susceptible to absorption of toxins through the skin (Moeller *et al.* 2003; Rahman *et al.* 2012), which may be another contributing factor.



Surprisingly, the presence of algae appeared to aggravate, rather that ameliorate, the effects of arsenic exposure in mosquitofish. In terms of increase in fish biomass, although algae acted antagonistically with arsenic, this resulted in a reduction in weight gained which is not likely to be advantageous. This effect is particularly apparent in smaller fish. For bioaccumulation the effects of algae were even more severe, as algae operated additively with arsenic to increase arsenic uptake and/or assimilation. Aggression was also highest in the presence of both algae and arsenic, although in this case the interaction was not significant. One plausible explanation concerns the biotransformation of arsenic by algae as described in the section 1 of this chapter. The exact nature of this transformation depends on algal growth and P nutrient status in the environment (Hellweger and Lall 2004; Levy *et al.* 2005; Rahman *et al.* 2012). Under P-limiting conditions, when algal growth is slow, algae excrete DMA^{III}. Under P-replete conditions with fast algal growth, PO_4^{3-} assimilation is up-regulated and As^{V} uptake increases in parallel. Since the transformation of As^{V} to As^{III} is faster than that of As^{III} to DMA^{III}, As^{III} builds up within algal cells and is consequently excreted into the environment to keep intracellular As^{III} at low levels and allow reductase activity (Hellweger and Lall 2004; Levy *et al.* 2005; Rahman *et al.* 2012). The phosphate concentration in our system was selected to simulate P-limiting conditions (Hellweger and Lall 2004; Moss 1998; Rahman and Hasegawa 2012) so should have limited algal growth and consequent arsenic uptake. However, as a recent study showed (Wang *et al.* 2013), even in P-limiting conditions algal As^{V} uptake may increase as cells synthesize more P transporters to compensate for the lack of phosphate in the environment. More importantly, however, fish metabolism produces waste, especially ammonia and phosphate. N and P recycling rates vary between species (Vanni *et al.* 2002; Villéger *et al.* 2012) and while the exact rate of N and P excretion by fish in this experiment was not quantified, stress is known to strongly stimulate urea (N) excretion in mosquitofish (Uliano *et al.* 2010). It is therefore likely that the presence of mosquitofish stimulated P-replete conditions and accelerated the biotransformation of arsenic by algae. A further consideration is algal growth. Nutrient supply, in particular phosphorus and nitrogen, is the most important determinant of algal production (Moss 1998; Rahman and Hasegawa 2012; Villéger *et al.* 2012). Algal growth, nutrient concentration, and arsenic are thus intricately linked. Research has shown a positive correlation between As^{III} concentration and primary productivity (Rahman and Hasegawa 2012) and the presence of fish is likely to contribute to this effect. Other elements such as oxygen (Smedley and Kinniburgh 2002; Wang *et al.* 2013) and iron (Senn and Hemond 2002) also influence arsenic speciation. Whatever the exact mechanisms here, it is evident that these various processes interacted to promote biotransformation of arsenic by algae. The end products of this transformation, in particular As^{III}, are less toxic to algae, but more toxic to fish (Rahman *et al.* 2012; Smedley and Kinniburgh 2002), so even if the overall aquatic arsenic concentration is reduced by algae, this may be counterproductive at an ecosystem scale.

For mosquitofish, the effects of arsenic exposure are overall detrimental. Despite the increased biomass seen here with arsenic, bioaccumulation of arsenic is harmful (de Castro *et al.* 2009; Moeller *et al.* 2003; Sopinka *et al.* 2010) and increased aggression may increase the

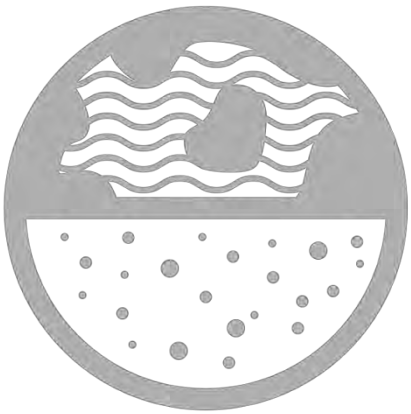
chance of physical damage (e.g. Huntingford and Turner 1987) and exacerbate physiological effects of arsenic exposure (e.g. Scott and Sloman 2004). Moreover, in addition to, or as a consequence of, the effects documented here other functions and interactions are likely to be disrupted. For example, both mate recognition (e.g. Fisher *et al.* 2006) and predator recognition (e.g. Mandrillon and Saglio 2007) are compromised by alteration of the chemical environment. The mechanisms underlying the behavioral changes demonstrated in this study may involve sensory, hormonal, neurological and metabolic systems (Scott and Sloman 2004) all of which may also affect other behaviors including locomotory behaviors like predator avoidance or swimming performance. The increase in aggression and lack of effects on feeding behavior in this study suggest locomotory functions were not affected. However, the exposure treatments here were neither particularly acute nor chronic and increased exposure concentrations or durations are likely to lead to more serious impacts. Finally, here we used an invasive, highly tolerant fish as a model. The effects of arsenic exposure on potentially endangered native species would be both more difficult and more critical to evaluate.

5. CONCLUSION

In conclusion, we have shown here that changes in complex behaviors are practical, ecologically relevant measures of toxicological effects. Aggression in particular should be considered in assessment of arsenic impacts as it is a highly dynamic and responsive process that may show immediate impacts and can influence several other aspects of behavior. In common with other authors, we also highlight interacting effects of contaminant exposure, both through integration of behavioral and physical mechanisms and consideration of different taxa together. Especially, toxicant responses in multi-trophic, natural ecosystems are often found to be different from single-species laboratory studies. Multi-trophic studies are therefore crucial to elucidate the real effects of toxicants. An important finding in this respect from the current study is the aggravating influence of algae on the impacts of arsenic exposure in fish. Bioremediation of arsenic contaminated waters using aquatic algae should therefore be carried out with consideration of entire ecosystem effects. Such multidisciplinary, cross-taxon research is crucial for understanding the impacts of arsenic toxicity and thus restoration of aquatic ecosystems.

CHAPTER 3

MUTUAL INTERACTION BETWEEN ARSENIC AND BIOFILM IN A MINING IMPACTED RIVER



Barral-Fraga L, Martiñá-Prieto D, Barral MT, Morin S, Guasch H.

Mutual interaction between arsenic and biofilm in a mining impacted river.

In prep.

ABSTRACT

Gold mining activities in fluvial systems may cause arsenic (As) pollution, as is the case in the Anllóns River (Galicia, NW Spain), where high concentrations in surface sediments (up to 270 mg kg^{-1}) were found. A 51 day-long biofilm translocation experiment was carried out in this river, moving colonized substrata from upstream (less As-polluted) to downstream the mine area (more As-polluted site with also more easily extractable As), to explore the effect of arsenic on benthic biofilms and the role of these biofilms on arsenic retention and speciation in the water-sediment interface. Eutrophic conditions (high total dissolved phosphorus and total nitrogen) were detected in water at both sites, while sediments were not considered P-polluted. Translocated biofilms accumulated more arsenic and showed higher potential toxicity (higher As/P ratio) than non-translocated ones. In concordance, their growth was reduced to half that observed in those non-translocated. Moreover, they became less nutritive (less N content) and with higher bacteria and dead diatom densities than the non-translocated biofilms. Methylated As-species (DMA^V) were found in the intracellular biofilm compartment and also in the river water, suggesting a detoxification process by biofilm (methylation) and its contribution to arsenic speciation in the water-benthic biofilm interface. Since most arsenic in sediments and water was arsenate (As^V), the high amount of arsenite (As^{III}) detected in the biofilm extracellular compartment may be attributed to As^V reduction by biofilms. Our study provides new arguments to understand microorganism contribution to arsenic biogeochemistry in freshwater environments.

1. BACKGROUND

Microorganisms constitute the majority of all living matter on Earth, most of them living in the form of multicellular aggregates commonly referred to as biofilms (Mora-Gómez *et al.* 2016). The modification of microbial composition and activity may have ecological consequences on local, regional and global scales (Huang 2014). In rivers, biofilms are the first to interact with dissolved substances from the surrounding environment, such as pollutants, being able to actively influence their sorption, desorption and transformation (Behra *et al.* 2002; Guasch *et al.* 2010). For all these reasons, fluvial biofilms provide an outlook of community ecotoxicology and allow a high degree of ecological realism either in ecotoxicological studies in micro/mesocosms or in the field by controlling the simultaneous exposure of many species and investigating direct and indirect toxic effects after acute and chronic exposure (Guasch *et al.* 2010). By carrying out studies in the field, the effects of pollution may be evaluated under real exposure conditions, using a set of biofilm parameters (i.e., endpoints) together with the analysis of water chemistry and the prevailing environmental conditions (Guasch *et al.* 2010; 2016). For instance, *biofilm translocation* experiments in fluvial systems using biofilm developed on artificial substrates are considered an active biomonitoring approach to assess the effects of metal pollution on these natural communities (Bonet *et al.* 2014; Morin *et al.* 2016). As a major component of benthic biofilms, diatoms (microscopic, unicellular brown algae) are considered

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4. General Discussion

Microalgae and prokaryotic communities play a key role on the biogeochemistry of arsenic. This has been extensively evaluated in marine systems and also in investigations focused on using biofilm for bioremediation of arsenic in groundwaters and, more recently, for biomining activity, a new mining practice consisting on the selective removal of arsenopyrite and pyrite by using acidophilic bacteria, facilitating the extraction of gold and silver (Drewniak and Sklodowska 2013). However, the implications of arsenic biogeochemistry in the ecology of freshwaters have been poorly addressed. This fact brings concern in terms of ecologic integrity since both arsenic detoxification and mobility have been attributed to microalgae and prokaryotic communities, the principal components of the fluvial biofilms.

The main objectives of this thesis were i) to contribute to the understanding of the interactions between arsenic and benthic biofilms in fluvial systems under environmentally realistic arsenic concentrations, ii) investigating the role of microorganisms (microalgae and bacteria) on the arsenic biogeochemical cycle, iii) identifying the toxic effects on the microorganisms, especially on diatoms, and the effects on their interaction with higher organisms (fish).

The naturally high arsenic concentrations in the water of some fluvial systems, such as those of the Pampa region in Argentina (130 µg As L^{-1}), have been chosen to develop the laboratory experiments of this thesis and to analyze the toxic effects to biofilms (***Chapter 1***) and higher organisms (***Chapter 2***) together, caused under these natural arsenic conditions. Otherwise, we also aimed to study the cycle and effects of arsenic when its pollution was originated by anthropic activities, including mining. In this case, arsenic is usually found in fluvial sediments, such as in the Anllóns River (***Chapter 3***), knowing that any physicochemical or biological change (e.g. phosphate entry or arsenic biospeciation) would cause an important release of arsenic into the water. The study of the As biogeochemistry is the key to understand both arsenic fate and toxicity.

The main results obtained from this thesis are summarized on Table 1, compared then to similar previous studies and discussed throughout this section, which was divided in four parts, encompassing: (1) the observed role of biofilm and environmental factors (especially, phosphate conditions) on arsenic biogeochemistry; (2) the arsenic toxicity detected in both biofilms and fish, and the influence of environmental factors on it; (3) the interaction between As-affected microbial communities and fish on nutrient cycling and arsenic toxicity; and finally, (4) we have tried to describe some perspectives and future research needs concerning these issues.

Table 1 Summary of the main results obtained in this thesis regarding the three main treatments (Tr.): Biofilm (B), Arsenic (As), and both Biofilm and Arsenic (B+As). [-P] indicates P-limited conditions; [+P] indicates non-P-limited conditions.

		"ACUTE" EXPOSURE (13 days)		**CHRONIC EXPOSURE (51 days)**	
Tr.	P (µg L^{-1}) **[ambient P conditions]**	***Chapter 1* (BIOFILM)** at 130 µg As L^{-1}	***Chapter 2* (FISH)** at 130 µg As L^{-1}	***Chapter 3* (BIOFILM and As-SPECIATION)** In Downstream at <2 µg As L^{-1}	
B	12.28 ± 3.34 [**-P**]	Higher *Fo* (total algal biomass) Main algal composition: **green algae** and diatoms	Lower fish agression Lower As bioaccumulation in fish Little weight gain (only on smaller fish)		
As	3.18 ± 1.17 [**-P**]		Higher fish agression Higher As bioaccumulation in fish Highest fish weight gain		
B + As	15.96 ± 4.14 [**-P**] in ***Chapters 1*** and ***2*** 190 [**+P**] in ***Chapter 3***	**Lower *Fo*; higher *Yeff* (diatoms)** **More heterotrophic biofilm** Main algal comp: **Diatoms (As resistance)** **Decrease diatom cell size** **Loss of diatom richness *(S)***	**AGGRAVATED EFFECTS OF As:** **Highest fish agression** **Highest As bioaccumulation in fish** Lower fish weight gain	***Fo* decrease** **More heterotrophic and less nutritive biofilm** **Higher dead-diatom density**	**SPECIATION** Sediment: As^{V} >>> As^{III} Water: As^{V} > As-Bet >> As^{III} ≈ **DMA^{V}** Intrac. biofilm: As^{V} > **DMA^{V}** >> As^{III} > As-Bet Extrac. biofilm: As^{V} > As^{III} > **DMA^{V}** >> As-Bet

1. ARSENIC BIOGEOCHEMISTRY

1.1 The biogeochemistry of arsenic observed in this thesis and the role of biofilms on it

Arsenic in freshwater systems is mainly found in sediments (Rahman and Hasegawa 2012) at mg kg^{-1} levels, as in our field experiment in the Anllóns River (***Chapter 3***); whereas low arsenic concentrations are usually found in the water compartment (at µg L^{-1} levels), with the exception of peculiar systems such as the Pampean rivers (e.g. Rosso *et al.* 2011). However, high arsenic concentrations can be desorbed from sediments and consequently released sporadically into the water (Magbanua *et al.* 2013) upon changes in environmental conditions (Rubinos *et al.* 2010), and also by microbial (epipsammic) activity (Garelick *et al.* 2009). Therefore, arsenic in water may be then available to other aquatic organisms and, in particular, to microorganisms such as the epilithic biofilm or periphyton. The activity of this kind of benthic biofilms may contribute to the mobility (and speciation) of arsenic in water. Actually, and in agreement with our hypothesis about arsenic mobility, we have verified the release of arsenic from sediments to other river compartments such as water and biota (***Chapter 3***). On the one hand, arsenic mobility from sediments to water have been demonstrated through two different ways; firstly, observing arsenic to be easily-extractable through exchange with phosphate and, secondly, detecting less arsenic concentration accumulated in the DGT device than that in the river water at the end of the experiment. Arsenic release from sediments through biological and physical disturbances has also recently been detected in other field study (Yan *et al.* 2016), suggesting to be the reasons of the seasonal variations of Total arsenic and As^{V} in the water of a freshwater system. On the other hand, arsenic mobility from water to epilithic biofilm or periphyton (and possible further excretion to water again) was also demonstrated in ***Chapter 3***, since the total arsenic concentration in biofilms exceeded the easily-extractable arsenic from sediments, thus suggesting arsenic accumulation over time and confirming that biofilm is a major sink for arsenic (López *et al.* 2016).

Additionally, and in agreement with our hypothesis about the contribution of fluvial biofilms to the arsenic speciation (***Chapter 3***), different arsenic species in biofilms have been detected when comparing to sediments, especially concerning methylated As-species, which were undetectable in sediments but especially elevated in biofilms (Table 1). The highest amount of methylated arsenic species was found in the biofilm intracellular compartment, in concordance with the biogenic origin of these organic species. Therefore, we can confirm that biofilms have intracellularly transformed iAs to orgAs species (DMA^{V}) through active methylation, probably as a detoxification process. Active methylation and detoxification processes in the intracellular compartment of epipsammic biofilms from the Anllóns River were described in the previous laboratory experiment by Prieto *et al.* (2016c) (Table 2). Finally, the presence of DMA^{V} in both water and biofilm compartments (lower concentrations in water than in biofilms) may indicate further mobilization from the biofilms to the water of this fluvial system.

Table 2 Summary of the main results obtained in a similar (and chronic) experiment to those of this thesis, where biofilm was exposed to arsenic: Prieto *et al.* (2016c). It is shown the main effects on biofilm only in a particular treatment (Tr.): Biofilm and Arsenic (B+As). Arsenic speciation in the arsenic exposed biofilm of this study is also reflected in the table. [-P] indicates P-limited conditions; [+P] indicates non-P-limited conditions.

CHRONIC EXPOSURE			
Tr.	**AMBIENT CONDITIONS**	**MAIN EFFECTS**	
B + As	22.82 µg P L^{-1} **[-P]** in **Prieto *et al.* (2016c)** at 0.98 µg As L^{-1} (water) and 106 mg As Kg^{-1} (sediment)	Main algal group: **cyanobacteria** Cyanobacteria (52%) > green algae (33%) > diatoms (15%)	
		SPECIATION	Sediment: mainly As^{V} Water: As^{V} >>> (As^{III} ≈ **DMA^{V}** ≈ **MMA^{V}** ≈ As-Bet) Intracellular biofilm (epipsammon): As^{V} >>> As^{III} > **DMA^{V}** > **MMA^{V}**

1.2 Discussing the influence of phosphate on the arsenic cycle in microorganisms

The role of phosphate on the cycle of arsenic by microorganisms is a controversial issue and there is still no consensus about the importance of P on the arsenic biogeochemistry, especially regarding microbial processes (uptake, speciation and excretion). In this thesis, we observed a relevant contribution of benthic biofilms to arsenic biogeochemistry through mobility and speciation processes (see Fig. 7 of ***Chapter 3***), which are very dependent on the epipsammic activity and on inputs of dissolved phosphate into the system. In consequence, different arsenic species (As^{V}, As^{III} and DMA^{V}) were finally detected in the studied biofilms.

Hellweger *et al.* (2003) proposed a model to explain the arsenic cycle in microalgae, depending on two conditions (see Fig. 3 of the ***General Introduction***): under P-limiting (-P) and non-P-limiting conditions (+P). In the first case (-P), little arsenic would be taken up by microalgae and methylation would be the main speciation process. In contrast, in the second case (+P), high amount of arsenic would be uptaken, and the principal pathway of arsenic cycle in microalgae would be the arsenate reduction to arsenite, followed by its further active excretion from the cell. Regarding Hellweger *et al.* (2003), the reduction of As^{V} to As^{III} by microalgae is fast under non-P-limiting conditions (+P), but methylation is slower, causing a peak of As^{III} in cells under these conditions and followed by its further high excretion. However, in this thesis (***Chapter 3***) methyl-As species resulted to be more relevant than As^{III} inside the cells under eutrophic conditions (Fig. 1), suggesting that methylation was a main process within these biofilms also under non-P-limiting conditions (+P).

The proposed model of Hellweger *et al.* (2003) oversimplifies the process of biospeciation by considering two cases (+P and –P) while, as it is reflected in the Table 3, it seems that an important variability of arsenic species may be found in algae or biofilms when they are exposed to different P concentrations, showing the complexity of the role of P on arsenic biospeciation. This lack of agreement in the literature may explain the confusion regarding the effect of P and the contradictory results that are found, especially in field studies. For instance, in their field study, Yan *et al.* (2016) put also into question the role of the total P on the biotransformation of arsenic. Their results suggest that the increase of the As^{III} mobility in water by microorganisms was mainly enhanced under low phosphate conditions, after As^{V} being rapidly taken up by microalgae via phosphate transporters and then reduced to As^{III}. Wang *et al.* (2013) have also detected that phosphorus limitation induces the reduction of As^{V} to As^{III}. Else, and contrary to the above-mentioned model again (Hellweger *et al.* 2003) but in concordance with our results (***Chapter 3***), production of methylarsenicals were especially detected inside of microalgal cells under eutrophic conditions (Yan *et al.* 2016). The remarkable production of methylarsenicals by freshwater green algae at high P concentrations was also demonstrated by Baker and Wallschläger (2016), calling also into question the conclusion made by some researchers that low P concentration is essential in the production of these organoarsenicals (e.g. Baker *et al.* 1983; Hasegawa *et al.* 2001; Hellweger *et al.* 2003). In conclusion, more studies regarding the influence of P on the biogeochemical cycle of arsenic species in aquatic environment are required, particularly in assessing the role of biofilm.

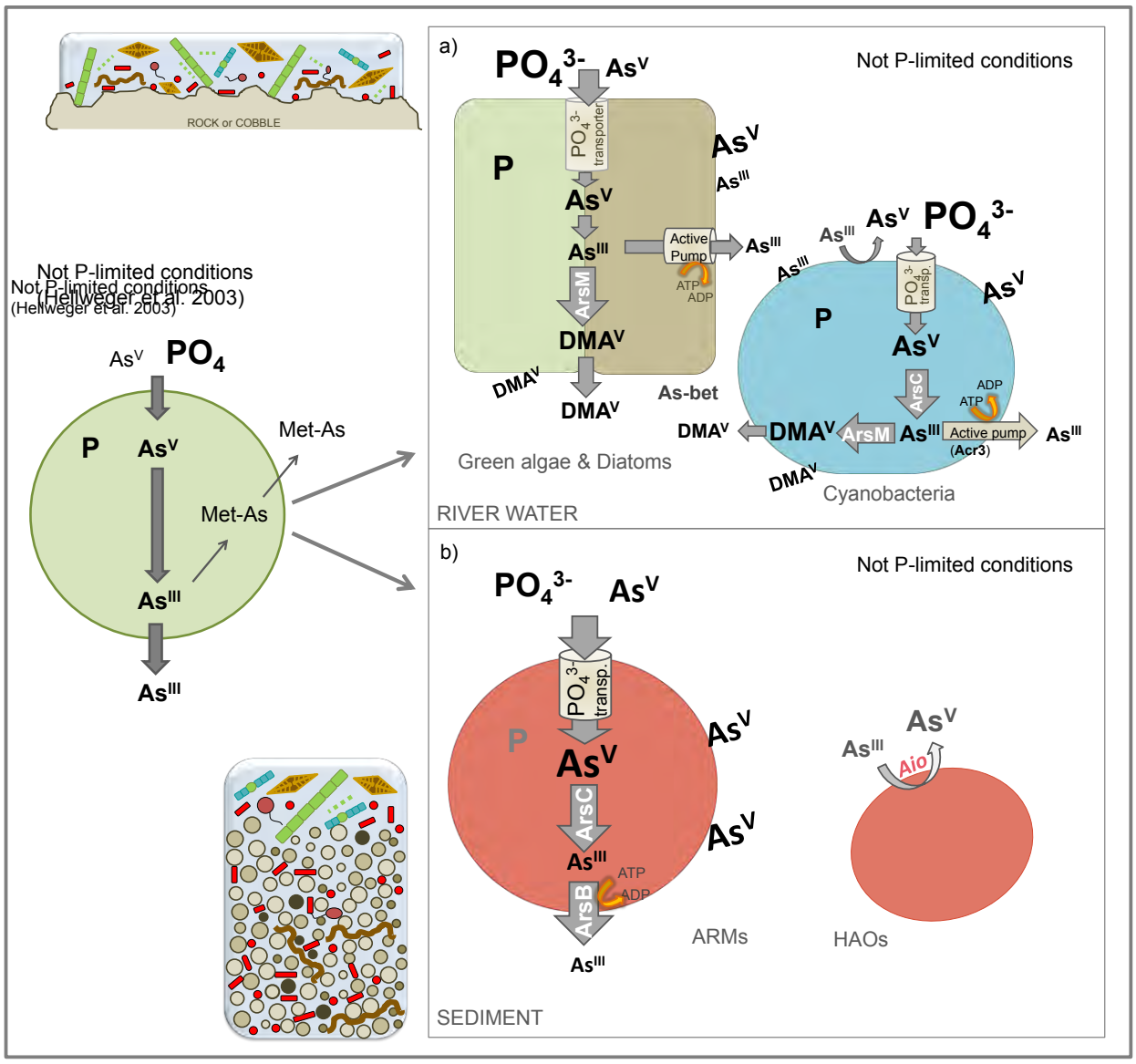

Figure 1 Hypothesized As-biospeciation processes by the fluvial biofilms described in the ***Chapter 3*** of this thesis, comparing with the model of Hellweger *et al.* (2003) under the same conditions (not P-limited): a) As-biospeciation by biofilms developed on artificial substrates simulating epilithic biofilms, and b) possible processes occurred by epipsammic biofilms hypothesized from the analysis of the arsenic species in sediments. The different font size of arsenic species represents their relative amount.

Table 3 Environmental arsenic speciation found in several studies after arsenic exposure to algae and/or biofilms, and under different environmental P concentrations ordered following the trophic classification (oligotrophic, mesotrophic and eutrophic; Dodds et al. 1998). Main arsenic species in the medium are shown (less important arsenic species found are in brackets). The data of this thesis (*Chapter 3*) correspond to the day 22 (90 µg P L^{-1}) and day 51 (190 µg P L^{-1}) after the biofilm translocation day.

Streams trophic classification (TP) by Dodds *et al.* (1998)	**Ambient [P] (µg L^{-1})**	**As sps. in the medium (mainly water)**	**References**
oligo-	0	As^{III}, As^{V}, DMA^{V}	Guo *et al.* (2011)
	1	As^{V}, DMA^{V}	Guo *et al.* (2011)
	10	As^{V}, DMA^{V}	Guo *et al.* (2011)
	<25	As^{III}	Baker and Wallschläger (2016) (similar to Guo *et al.* 2011 at 0 µgP L^{-1})
meso-	25	DMA^{V}	Baker and Wallschläger (2016)
	40	As^{V} (As^{III}, DMA^{V}, MMA^{V})	Yan *et al.* (2016)
eu-	90	As^{V}	This thesis *(Chapter 3)*
	150	As^{III}	Levy *et al.* (2005)
	>150	DMA^{V}	Baker and Wallschläger (2016)
	175	As^{V} (As^{III}, DMA^{V})	Guo *et al.* (2011)
	190	As^{V} > As-Bet >> As^{III} ≈ DMA^{V}	This thesis *(Chapter 3)*
	220	As^{V} (As^{III}, DMA^{V}, MMA^{V})	Yan *et al.* (2016)
	1500	As^{III}	Levy *et al.* (2005)

1.3. The influence of other environmental factors on the arsenic biogeochemistry in freshwaters

Besides phosphorus, other environmental factors (both physicochemical and biotic) may modulate the arsenic cycle in freshwaters.

Arsenic remobilization due to physicochemical or indirect biological processes

Not only high levels of phosphate but also of dissolved organic matter enhance arsenic release from sediments as a result of competition for adsorption sites (Smedley and Kinniburgh 2002). That is, high DOC may also promote the mobility and bioavailability of arsenic (Yan *et al.* 2016). Moreover, arsenic release from sediments can also be due to a decrease of the redox potential (Eh) and dissolved oxygen at the sediment–water interface caused, for instance, by the effect of increased temperature on microorganisms activity (vigorous microbial activities are usually observed during warm seasons in eutrophic environments) (Hasegawa *et al.* 2009; Yan *et*

al. 2016). Increased pH observed during photosynthetically active summer algal blooms may also induce arsenic release from the sediments (Dzombak and Morel 1990). In addition, some physical factors such as strong hydraulic turbulence, affected by wind, may promote the arsenic liberation from sediments (Wei *et al.* 2011).

Changes in arsenic speciation due to physicochemical or biological processes

Several physicochemical and biological factors may cause changes in arsenic speciation. For instance, arsenic biomethylation was found to be conditioned by water temperature and light intensity (Karadjova *et al.* 2008). Also, biotransformation of methylarsenical to more complex organoarsenic compounds in warm seasons was also detected (Hasegawa *et al.* 2009, 2010; Yan *et al.* 2016). Due to the large capacity of microalgae to bind trace elements, an excessive growth of microalgae enhanced by high temperature and/or eutrophic conditions may lead to accumulate a large amount of arsenic in the aquatic environment (Radway *et al.* 2001) and, as algal blooms may alter the pH and redox potential and affect the concentrations of DOC and Fe/Mn compounds (Eggleton and Thomas 2004), they finally may strongly influence the chemical forms of arsenic in the aquatic environment. Finally, the decrease of dissolved oxygen contents, which may be associated to vigorous bacterial activities and/or the decomposition of algal blooms, may contribute to the change of arsenic speciation benefiting, for instance, the presence of As^{III} in the water (e.g. through anaerobic arsenate respiration) (Baeyens *et al.* 2007; Hasegawa *et al.* 2010).

The influence of other biotic drivers

The use of different species of algae may also be the cause of the observed discrepancies in freshwater arsenic biotransformation. As suggested by Qin *et al.* (2009), varying levels of methylation observed in previous laboratory cultures could be partially due to differences in the activity of arsenic methyltransferases between species. Therefore, more work is needed to determine a threshold of arsenic methylation in different algal species (Baker and Wallschläger 2016).

Furthermore, it is important to note the differences in arsenic speciation between cultured microalgae and microalgae collected from the natural environment, that might be explained by the simultaneous collection of sediments or particles containing inorganic arsenic within the samples of microorganims (Caumette *et al.* 2012), as biofilm samples. A probable consequence of this fact may be to overlook the production of arsenosugars by microalgae in the natural environment, because their presence may result to be obscured by the predominant presence of iAs (Caumette *et al.* 2012).

2. ARSENIC TOXICITY

In agreement with our hypothesis about toxicity, we found that arsenic affects biofilms, causing changes in their function and structure (***Chapter 1***); however, we did not expect the detected contribution of biofilms in the aggravated toxicity of arsenic to higher organisms as fish (***Chapter 2***).

2.1. Arsenic toxicity to biofilms

We detected microalgae to be heavily affected by arsenic exposure as reflected in the loss of biomass and functionality, especially in photosynthesis; while increased bacterial densities were found as a response of arsenic exposure. Therefore, in this thesis (especially in ***Chapter 1***), arsenic caused biofilms to become less autotrophic or phototrophic and thus more heterotrophic, reflecting the toxic effects to microalgae and the highly documented bacterial resistance to this metalloid. The loss of microalgal biomass was found under both acute arsenic exposure (130 µg As^{V} L^{-1} during 13 days, in ***Chapter 1***) and chronic low-dose exposure (ranging from 0.79 to 1.83 µg As L^{-1} during 51 days, in ***Chapter 3***).

Similar effects on biofilms were described in other studies (Table 4) such as the chronic arsenic exposure (120 µg As L^{-1} during 60 days) in Tuulaikhuu *et al.* (2015), where photosynthetic activity was also inhibited, becoming biofilms less phototrophic. In their study, arsenic concentrations decreased from 120 to 28 µg As L^{-1}, due to most arsenic finally sunk to the sediment and a smaller percentage accumulated in the biofilm. Less phosphate was also found (ranged from 5 to 6 µg PO_4^{3-} L^{-1}) than in our studies (around 16 µg PO_4^{3-} L^{-1} in ***Chapter 1***; and around 190 µg PO_4^{3-} L^{-1} in ***Chapter 3***). In another chronic experiment (Rodriguez-Castro *et al.* 2015), where biofilms were exposed to 15 and 130 µg As^{V} L^{-1}, inhibition of algal growth and photosynthetic capacity was also detected, as well as change of algal community composition and reduced ability of the community to retain P. These effects on biofilms were detected in P starved communities (below 10 µg PO_4^{3-} L^{-1} in medium), showing that chronic exposure to arsenic led to changes in the photosynthetic apparatus exclusively under conditions of P limitation, which agrees with our results in ***Chapter 1***, but not when P-availability was higher (100 µg PO_4^{3-} L^{-1}), disagreeing with the results obtained in ***Chapter 3***. However, biofilms in our field experiment of ***Chapter 3*** were closely located to very high arsenic concentrations in sediments (higher than in the previous described studies). Another difference with the laboratory experiments of Rodriguez-Castro *et al.* (2015) and Tuulaikhuu *et al.* (2015) may be the probable contribution of other environmental factors (and even a combination of them) to the observed responses in these microorganisms.

Since the different species of microalgae found in a biofilm may differ in their metal and metalloid sensitivity, it is expected that high concentrations under conditions of acute exposure, and even low concentrations during long-term exposures, may lead to modifications in their competitive interactions, producing community changes (Serra *et al.* 2009), as was especially observed in ***Chapter 1*** (acute exposure in laboratory experiment), where only diatoms dominated after 13 days of arsenic exposure.

Table 4 Summary of the main results obtained in similar (and chronic) experiments to those of this thesis, where biofilm or/and also fish were exposed to arsenic (As): Tuulaikhuu *et al.* (2015, 2016) and Rodriguez-Castro *et al.* (2015). Treatments (Tr.) are Arsenic (As), and Biofilm with Arsenic (B+As). The main effects of arsenic in biofilm and/or fish are shown, comparing with their controls (biofilm and fish without arsenic exposure of each experiment). [-P] indicates P-limited conditions; [+P] indicates non-P-limited conditions.

CHRONIC EXPOSURE		
Tr.	**AMBIENT CONDITIONS**	**EFFECTS (in biofilm or fish)**
As	5 – 6 µg P L^{-1} **[-P]** in **Tuulaikhuu *et al.* (2016)** (**FISH**) at 34-40 µg As L^{-1}	Higher fish weight gain (only in larger bodies) than in their treatment **B** (only biofilm and fish)
B + As	5 – 6 µg P L^{-1} **[-P]** in **Tuulaikhuu *et al.* (2016)** (**FISH**) at 34-40 µg As L^{-1}	**NO AGGRAVATED EFFECTS OF As EXPOSURE BY BIOFILM IN FISH**
	5 - 6 µg P L^{-1} **[-P]** in **Tuulaikhuu *et al.* (2015)** (**BIOFILM**) at 120-28 µg As L^{-1}	COMPARING WITH THEIR TREATMENT **B (only biofilm)**: Lower total biomass (*Fo*) Inhibition diatom growth Lower N content (less nutritive biofilm) Epipsammon: more heterotrophic
	10 µg P L^{-1} **[-P]** in **Rodriguez-Castro *et al.* (2015)** (**BIOFILM**) at 130 µg As L^{-1}	COMPARING WITH THEIR TREATMENT **B (only biofilm)**: Changes in photosynthetic apparatus Inhibition of algal growth Changes in diatom sizes Reduction in the diatom relative abundance Diatom community adaptation (As resistance)
	100 µg P L^{-1} **[+P]** in **Rodriguez-Castro *et al.* (2015)** (**BIOFILM**) at 130 µg As L^{-1}	**NO EFFECTS OF As IN BIOFILM AT [+P] CONDITIONS**

Abiotic and biotic factors that may modulate arsenic toxicity to biofilms

Arsenic uptake and toxicity to microorganisms may be influenced by abiotic and biotic factors such as pH and redox potential of the medium, temperature, light intensity and availability, nutrient availability, but also the production of extracellular polymeric substances (EPS) and metal speciation (Karadjova *et al.* 2008; Letovsky *et al.* 2012). However, operational separation of biotic and abiotic influences is difficult due to their interactive nature in biofilms. For example, the physicochemical-binding capacity of periphytic mats can be enhanced by benthic microalgae through biologically mediated processes, such as production of EPS,

dissolved oxygen and the maintenance of pH conditions which are, in turn, influenced by abiotic factors like nutrient and light availability (Letovsky *et al.* 2012). Actually, in our field experiment (***Chapter 3***), not only arsenic but also other environmental factors such as light availability or intensity could have influenced the observed effects on biofilms, as it was already detected by other authors studying the positive effect of light (until 500 µmol s^{-1} m^{-2}, since photoinhibition was detected above this light intensity, e.g. Villeneuve *et al.* 2010) and biomass on the uptake and sorption of some metals by biofilms (e.g. Gray and Hill 1995; Garnham *et al.* 1992; Hill *et al.* 2000; Gray *et al.* 2001). However, these findings about the influence of light on metal sorption do not agree with our results in ***Chapter 3***, since higher arsenic concentrations in biofilms (and sediments) were found in the site with higher riparian cover. On the other hand, the variation in light intensity, in comparison to constant light required in well-designed toxicity test, could have measurable consequences on photosynthesis of natural biofilms and therefore on their response to toxicants, as suggested by Laviale et al. (2010). In their study, biofilms demonstrated a relative plasticity in their photobiology in response to the large variation of light encountered during the experiment (up to 1000 µmol s^{-1} m^{-2}), and they finally concluded that a dynamic light regime increased periphyton sensitivity to toxicans (e.g. isoproturon) when comparing under constant light regimes, probably by challenging its photoprotective mechanisms such as the xanthophyll cycle. Taking into account that the site with higher riparian cover in the ***Chapter 3*** (Down site) was also exposed to higher light variation during time (see values of "Light reaching biofilm" in Table 1a of ***Chapter 3***) than in the site with less riparian cover, we can tentatively conclude that this light variation could be an important factor that increased biofilm sensitivity to arsenic, resulting in more affected biofilms in this Down site. In other cases, neither light nor biomass have been detected to influence metal accumulation in periphytic biofilms (e.g. Letovsky *et al.* 2012).

2.2 Diatom responses to arsenic exposure

The identification of arsenic effects in our biofilms was especially focused on diatoms. Contrary to what several authors highlight about the high sensitivity of diatoms to toxicants among the aerobic photosynthetic microorganisms (e.g. Hill 1996; Corcoll 2011; Prieto *et al.* 2016c), we have found diatoms to evolve arsenic adaptation under arsenic "acute" exposure (***Chapter 1***), and becoming more resistant to this metalloid than other microalgal groups (green algae and cyanobacteria). However, this resistance had a cost: a clear decrease of real cell size or cell biovolume and a slight loss of species richness (*S*) (***Chapter 1***). Changes in diatom sizes were already announced by Rodriguez-Castro *et al.* (2015) as an expected effect of arsenic exposure. Cell size decrease in diatoms exposed to high metals was previously detected (e.g. Cattaneo *et al.* 1998; Cattaneo *et al.* 2004; Morin and Coste 2006; Luís *et al.* 2011; Menció *et al.* 2016, see Fig. 2) but in some studies theoretical biovolume data are used instead of performing real cell measurements. Taking real measurements for cell biovolume is time consuming but, in this thesis (***Chapter 1***), we demonstrated clear differences comparing real with theoretical measurements. We also demonstrated that, contrary to what some authors

suggested (e.g. Lavoie *et al.* 2006), real cell size provides strong additional and clear information about diatom responses to environmental toxicity. The reasons of this size decrease could lie in a higher cellular division rate during vegetative reproduction, typically under stressed conditions (Morin *et al.* 2012). However, several environmental factors could also contribute to the cell size decrease on diatoms and other microorganisms, as already commented in the ***General Introduction*** (sub-section 2.5).

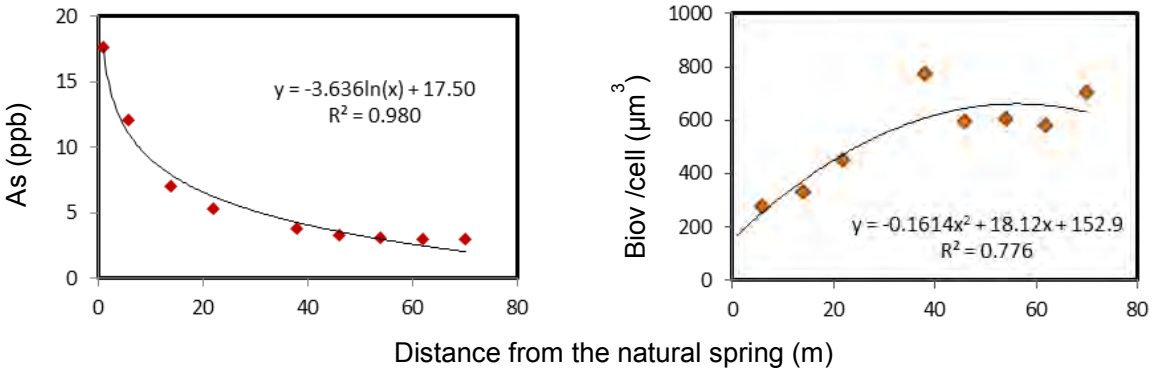

Figure 2 Polynomic fitting curve for environmental arsenic concentrations and diatom biovolume (μm^3) per cell parameters is shown, in a field study where aquatic arsenic was gradually decreasing from the natural Can Verdaguer spring. From Menció *et al.* 2016.

Ecological impact of cell size decrease

It has been suggested that the size of organisms at any trophic level in the aquatic environment can be a determining factor in the ecological efficiency of energy transfer, as well as in the type of organisms living at the highest trophic level. For instance, the yield of fish from a marine ecosystem predominated by phytoplankton with large cells was found to be much greater than that from areas predominated by phytoplankton with small cells (Parsons and Takahashi 1973). In this thesis, we observed that arsenic may decrease the cell size of the diatom community, a main component of the biofilm (***Chapter 1***), as well as to transform biofilm from a N-rich to a poorer (high C/N ratio) composition (***Chapter 3***). Regarding the review of Finkel *et al.* (2010), cell size and elemental stoichiometry often respond predictably to abiotic conditions and follow biophysical rules that link environmental conditions to growth rates, and growth rates to food web interactions and, consequently, to the biogeochemical cycling of elements. Moreover, it was observed that the size structure and elemental composition of the phytoplankton community may have a cascading influence on the proportion of organic material transferred to the microbial loop and higher trophic levels (Finkel *et al.* 2010). Therefore, since a shift is predicted towards smaller phytoplankton species caused by the global change, leading to a cascading negative effect on the productivity and size structure of the benthic food web (Finkel *et al.* 2010), it could be also predicted similar consequences on As-affected ecosystems regarding, for instance, the effects of the diatom cell size decrease.

2.3. Arsenic toxicity to fish

Fish can uptake trace metals by two main routes (Farkas *et al.* 2003; Terra *et al.* 2008; Rozon-Ramilo *et al.* 2011), either by adsorption from water through the gills, and from food absorbed through the digestive tract. The predominant pathways for metal uptake appear to be highly variable over the range of metals, fish species and levels of contamination. The bioavailability and further bioaccumulation of metals in fish depends, thus, on the concentrations in water and the rest of the ecosystem, such as biofilm, invertebrates and sediment, being the last one frequently ingested with food by bottom feeders. Nevertheless, direct proportionality does not necessarily exist between water concentrations and bioaccumulation levels in aquatic organisms (Andres *et al.* 2000; Yi and Zhang 2012). Arsenic toxicity to fish may be studied using a wide variety of biomarkers ranging from, for instance, molecular analyses such as enzyme activity determination (e.g. Tuulaikhuu *et al.* 2016) to analyses related to fish physiology and behavior (e.g. ***Chapter 2***). In this thesis (***Chapter 2***), fish exposed to dissolved arsenic (130 μg L^{-1} or ppb) have become more aggressive and also have increased their weight, as well as their arsenic tissue content (around 600 μg g^{-1} or ppb). However, the highest arsenic bioaccumulation (almost 800 μg g^{-1}) and strongest aggression in fish (leading to a decrease of their weight gain) were detected in our study when analyzing their interaction with biofilms, showing the importance of including different trophic levels together on As-impact studies (see below in sub-section 3, about interactions). Several studies have detected biochemical changes and genotoxicity effects on fish due to arsenic exposure (e.g. Castro *et al.* 2009; Ventura-Lima *et al.* 2009; Kumar *et al.* 2014; Tuulaikhuu *et al.* 2016), with concentrations ranging from 10 to 100 μg As L^{-1}. Under lower aquatic concentrations (around 2 μg As L^{-1} in water) but with higher values in sediments (ranging from 10 to 14 mg kg^{-1} or ppm), consistent negative relationships between fish size and environmental arsenic concentrations was detected in different fish species (the small-sized bleak *Alburnus alburnus* and the Languedoc gudgeon *Gobio occitaniae*, as well as the large-sized Ebro barbel *Luciobarbus graellsii*), which are gregarious species feeding both on plant material and macroinvertebrates, searching for food mainly on the river bottom (Ebro barbel, gudgeon) and in the water column (especially bleak), and which have reached to bioaccumulate up to 5.6 mg As kg^{-1} or ppm (average value) in their muscles (Merciai *et al.* 2014). Therefore, we may conclude that arsenic may cause toxicity to different fish species at aquatic concentrations from 2 to 130 μg L^{-1}, but the presence of other ecosystem compartments such as microorganisms and sediments (e.g. Merciai *et al.* 2014; Tuulaikhuu *et al.* 2016) influences on the fish responses to this arsenic exposure. Regarding arsenic species, values for fish from the ECOTOX database are set at higher concentrations (Tuulaikhuu 2016), setting the LC50 values at 40.9 mg As^{V} L^{-1} and 24.5 mg As^{III} L^{-1}.

2.4 Biomarkers of arsenic toxicity used in this thesis

In view of our results, we recommend some methods for further use as biomarkers of arsenic toxicity to biofilms and fish, understanding biomarkers in stress ecology as functional

measures of exposure to chemical and physical disturbances that give information on the upper biological organization level (Guasch *et al.* 2017).

For instance, the chl-a fluorescence techniques such as the Pulse-Amplitude-Modulated (PAM) fluorescence may be considered an optimal biomarker to monitor the influence of arsenic effects on microalgae photosynthesis and evaluate both functional and structural alterations in the autotrophic biofilm communities. Using the PAM-florescence allowed to successfully detect metal pollution in biofilms in previous studies (e.g. Navarro *et al.* 2002; Guasch *et al.* 2003; Bonnineau *et al.* 2011).

We also strongly recommend performing real measurements of the diatom cell biovolume and including it as a biomarker of arsenic toxicity, despite some limitations that it entails. Proposals to improve this technique are given in sub-section 4.1 "**Diatom future perspectives**".

Concerning fish, we found that changes in complex behaviors such as aggression may be considered as practical biomarkers of arsenic toxicity, since aggression is a highly dynamic process that may give responses to an immediate and ecologically relevant impact. Increasing aggression in several fish species due to arsenic toxicity was detected in some previous studies (e.g. Moss 1998; Scott and Sloman 2004; Weis*et al.* 2001), and we suggest to still incorporate it in ecotoxicological impact studies.

3. THE BIOTIC INTERACTIONS BETWEEN MICROBIAL COMMUNITIES AND FISH

Effects of contaminants may occur at all levels of organization, from molecular to ecosystem-level responses, but there is no single spatiotemporal scale or level of biological organization at which ecotoxicological investigations should be conducted (Clements 2000). Biochemical and physiological alterations in organisms may occur rapidly and are often stressor-specific. However, while these alterations in populations and communities have greater ecological relevance, a firm mechanistic understanding of these responses is often lacking. Therefore, developing mechanistic linkages across levels of biological organization would greatly improve our understanding of how organisms are affected by contaminants in nature (Clements 2000). Moreover, the failure of traditional toxicity tests to consider dietary exposures and trophic transfer of contaminants is a recurring theme in discussions of the limitations of such tests' translation to nature (Buchwalter *et al.* 2017). Regarding Buchwalter *et al.* (2017), a common misperception is that diet-derived metals are not toxic relative to dissolved exposures and, in fact, dietary toxicity studies in aquatic organisms are relatively rare, in part because they are minimally supported by regulatory entities. Such studies would be significantly more informative than water-only exposures (Buchwalter *et al.* 2017).

Biofilms are the basis of the trophic web in rivers, being important sources of energy for invertebrates and herbivorous fish (Stevenson *et al.* 1996). Moreover, biofilm functioning influences the main processes in streams (Romaní *et al.* 2004). Actually, the fluvial biofilm that

covers the river bed is an active place, exerting an important ecosystem service by participating in its purification through capturing excess nutrients and degrading or transforming many contaminants. Then, the effects of pollutants on primary producers may ramify through ecosystems because they provide food for higher trophic levels and mediate the biogeochemical cycling of nutrients and contaminants (Hill *et al.* 2010). Therefore, the content of pollutants like metals and metalloids in benthic biofilms is a means of evaluating the state of contamination in the environment with respect to levels of metals and metalloids, and their bioavailability (Behra *et al.* 2002).

In this thesis, we have studied the interactions between biofilm and fish (***Chapter 1*** and ***2***) as a means of evaluating, with greater ecological realism, the possible effects of arsenic in fluvial systems. Under natural conditions, the interaction between biofilm and fish is related to the nutrient cycling, which is a crucial process in the ecosystem functioning. Actually, fish play an important role as nutrient subsidies, while biofilm actively uptakes the nutrients, playing a role in water purification and increasing productivity in the subsidized system. However, arsenic may change nutrient dynamics and, finally, influence the whole ecosystem (Tuulaikhuu 2016).

3.1. The influence of fish on arsenic toxicity to algae

The expected role of fish in this thesis was to provide a supply of P in the system, through fish excretion. Specifically, we expected higher fish excretion due to arsenic stress, what would provide enough phosphate in the medium to protect biofilm against arsenic toxicity (less arsenic would be uptaken by microorganisms due to its competition with phosphate). However, aquatic P resulted to be limited even in the presence of both fish and As, and strong toxicity was observed in As-exposed biofilms (***Chapter 1***), what could indicate that fish excretion (not measured) was not enough to protect the biofilms (Fig. 3). Hence, on one hand, we may predict that, due to the P-limited conditions in our system, microalgae would synthesize more P transporters to compensate for the lack of environmental P (Wang *et al.* 2013), what would lead to an increase of arsenic uptake by biofilms. However, on the other hand, despite detecting low P concentrations in the water (***Chapter 1*** and ***2***), high chl-a concentrations in biofilms were found. These concentrations of chl-a corresponded to eutrophic and mesotrophic-eutrophic conditions (even when biofilm was under arsenic exposure) following the boundary of Dodds *et al.* (1998) for rivers (max. benthic chl-a concentration for mesotrophic-eutrophic boundary set at 20 μg cm^{-2}). Taking into account that a nutrient is considered limiting when it causes limitation of primary production (Margalef 1983), and that the chlorophyll-a standing crop is contemplate as an estimation of primary production, the algae did not really seem to be starved in ***Chapter 1*** and ***2***, since their standing crop indicated no nutrient limitation for them, suggesting that phosphate in water would correspond to the remaining concentration after being uptaken by microalgae. Accordingly, besides environmental phosphate, intracellular P should have also been analyzed to know the real nutrient status of the cells and better interpret the arsenic cycle in them. Therefore, in non-P-limited environments, if cells are damaged by arsenic toxicity, they may probably not uptake enough P for primary production, as observed in Rodriguez-Castro *et*

al. (2015); while a P-limited environment may be a consequence of a previous P uptake by algae.

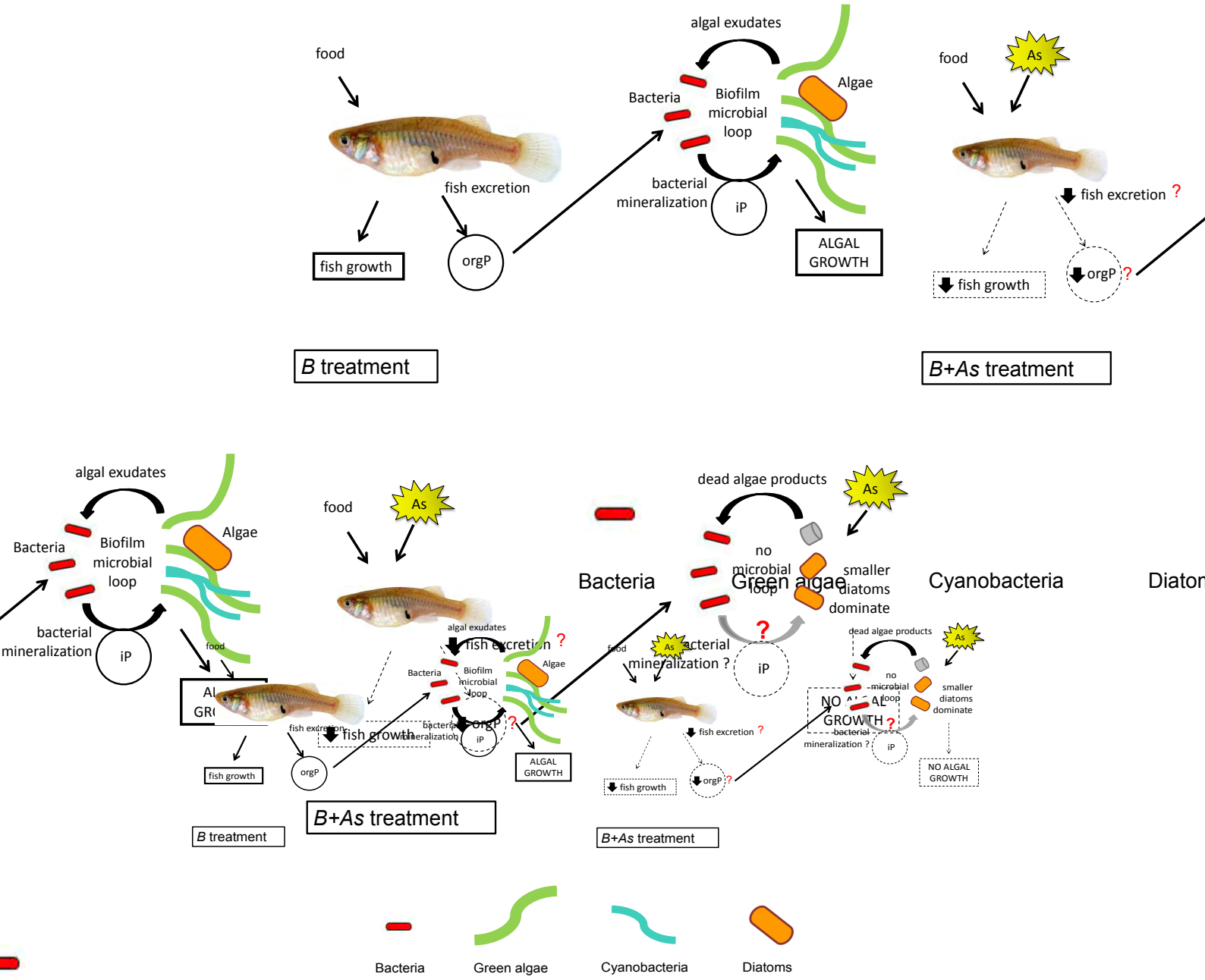

Figure 3 Theoretical-Model of the interaction between fish and biofilm studied in this thesis, comparing a situation under arsenic exposure (*B+As* treatment) with no arsenic exposure (*B* treatment). orgP: organic phosphate. iP: inorganic phosphate. As: arsenic exposure. Dotted lines represent decreased or ceased processes.

3.2. The influence of biofilm on the arsenic toxicity to fish

In spite of having used an invasive and highly tolerant fish species in our experiments,

the Mosquitofish, arsenic toxicity was anyway detected in them. Moreover, and surprisingly, effects in fish were aggravated with the presence of biofilm, resulting in higher stress and physiological changes, as well as higher arsenic accumulation in their tissues than under arsenic exposure without biofilm (***Chapter 2***). Opposite effects were detected in a similar experiment (Tuulaikhuu *et al.* 2016), where biofilms have temporally reduced arsenic toxicity to fish (Mosquitofish) under limiting P conditions, demonstrating their role on water purification. In Tuulaikhuu *et al.* (2016), similar arsenic concentration was used, but double fish density was choose comparing with our experiment and, moreover, a sediment component was added (located in other tank separately from biofilms), proving to be responsible for arsenic retention (since arsenic concentration resulted finally almost 2 times lower in water than in ***Chapter 2***), and also for P retention (resulting in less dissolved P than in our experiment). Besides lower arsenic concentration, differences in P availability and thus arsenic speciation could explain why biofilm increased arsenic toxicity to fish in our experiment (***Chapter 2***) but decreased it under lower aquatic P concentration (Tuulaikhuu *et al.* 2016). Having this in mind, we may hypothesize that the aggravated effects of arsenic on fish were probably caused through excretion of the highly toxic As^{III} into the water by biofilms, or through the production and further excretion of methylarsenicals such as DMA^{III} and MMA^{III}, that are even more toxic than the inorganic As^{III}. Unfortunately, speciation was neither analyzed in this ***Chapter 1*** nor in ***Chapter 2*** and, as already explained, it is still uncertain how PO_4^{3-} influences the production of arsenic species in microalgae. Another possible reason of this increased toxicity may lie in the fact that biofilms (especially, periphyton) are an important intermediary for the fate of biomagnified contaminants from the water to fish (Hill *et al.* 2010). Therefore, the higher arsenic accumulation in fish could probably also be due to some biofilm ingestion since, despite being biofilm and fish located separately in different compartments, a transfer of self-detached biofilm with water flow was sometimes observed and fell down to fish compartment, being this effect already observed and considered in previous studies (e.g. Boulêtreau *et al.* 2006). Given the “age” of the biofilm, and the fact that it was “stressed” by As, it could be possible that autogenic detachment occurred during our experiment. Moreover, it is common to find metal accumulation in the dead cells of the upper layers of biofilms (those likely to detach) (Teitzel and Parsek 2003) and it may also be expected for metalloids, what would contribute to the availability of high arsenic concentrations for fish, leading probably to the detected higher arsenic accumulation (and aggravated effects) in their tissue. Definitively, the aggravating influence of biofilms on the impacts of arsenic exposure in fish was an important finding that, on one hand, shows the eventual loss of its role on water purification (depending presumably on the P conditions) and, on the other hand, manifests the importance of multi-trophic studies in ecotoxicology to elucidate the real effects of toxicants from an ecosystem perspective.

4. FUTURE PERSPECTIVES AND RESEARCH NEEDS

4.1 Diatom future perspectives

Towards easier and feasible diatom endpoints

Diatoms are regularly used for bioassessment and ecotoxicological studies in relation to environmental and anthropogenic disturbances. Traditional taxonomical diatom parameters such as cell counts, biovolume estimates, species richness, diversity indices and metrics using sensitive and tolerant diatom species are regularly used for these studies (Pandey *et al.* 2017). Several diatom endpoints were studied in this thesis, as the changes in the community structure due to its sensitivity to arsenic exposure, detecting a selection for As-tolerant species, in particular *Achnanthidium minutissimum*. Species richness was also a good endpoint in our ecotoxicological studies. The use of these traditional community structural metrics using diatoms may provide effective diagnostic information about fluvial ecosystems' health. However, the extensive time (and financial) requirements, the necessity of expertise in diatom taxonomy and the need for statistical validation means that the use of structural metrics often make these metrics not feasible (Pandey *et al.* 2017). Exploring new endpoints, along with the traditional taxonomical parameters, can greatly enhance the evaluation of fluvial ecosystem quality for biomonitoring practices (Pandey *et al.* 2017). In this sense, new endpoints are nowadays also applied due to their numerous merits, like their easiness, quickness, cheapness, global acceptation and no especial training in diatom taxonomy (Pandey *et al.* 2017). For instance, life-forms (e.g. Rimet and Bouchez 2011); alterations in cell integrity, including nuclear anomalies (e.g. Licursi and Gómez 2013), alterations in the cell membrane, cytoplasmic content and photosynthetic apparatus (e.g. Wood *et al.* 2014); cell motility (e.g. Coquillé *et al.* 2015), changes in number and biovolume of lipid bodies (e.g. Pandey and Bergey 2016), size reduction and deformities or teratologies (already commented). All these new endpoints form a very promising basis for easy and rapid ecological assessments as well as for biomonitoring of fluvial ecosystem (Pandey *et al.* 2017). In this thesis, size reduction may be considered a mixture between traditional and new endpoint since the measures were done after diatom taxa identification, to almost all species found, which has involved greater difficulty.

The cell measurements of all species found in the samples of the ***Chapter 1*** were really time-consuming. A good alternative method would be to perform measurements only on selected species that would be abundant enough in the different samples (contaminated and non-contaminated) to get robust differences. Actually, it was a method successfully applied on other studies such as in Morin and Coste (2006). We also expect future facilities in technology for measurements using, for instance, flow cytometry with a high resolution camera incorporated, giving specific cell biovolume and images (since maximal resolution camera on flow cytometry is nowadays limited for big diatoms).

New endpoints such as live-cells density and observation of teratologies were used in this thesis and significant decrease of live cells in our field experiment (***Chapter 3***) was

observed. The absence of significant amounts of teratologies (morphological alterations) in diatoms exposed to arsenic in ***Chapter 1*** is very remarkable, since this is totally in contradiction with what happens with diatoms exposed to some metals such as cadmium and zinc (e.g. Morin 2006; Pandey *et al.* 2014). But, undoubtedly, the most significant detected endpoint in this thesis was the change on real specific cell size, seeing a clear reduction in the average size on As-exposed communities. In their review about diatom teratologies, Falasco *et al.* (2009) consider the change in size as a type of morphological alteration in diatoms (teratology type 3). Therefore, we can conclude that metalloids like arsenic may also cause teratological forms of diatoms.

Towards molecular tools for diatom identification

Diatom taxa identification is nowadays based on the morphology of their silica skeleton (frustule), and hundreds of valves under the light microscope are usually counted and determined as standard procedures of diatom biomonitoring in water quality assessment. However, as explained above, this is time-consuming and requires a high level of taxonomic expertise, especially to distinguish morphologically very similar taxa and avoid misidentifications. Molecular identifications based on DNA sequences are expected to be an alternative in diatom taxa identification that reduces analysis time and could avoid uncertainties (Rimet *et al.* 2016). The DNA-barcoding is a taxonomic method that uses a short genetic marker in an organism's DNA to identify it as belonging to a particular species (Hebert *et al.* 2003). Primers specific to diatoms have been proposed (Valiente Moro *et al.* 2009) but they are not specific enough to characterize the real diversity in diatom communities (Morin *et al.* 2012).

Barcoding applied to natural samples with several taxa is named metabarcoding, and the development of Next-Generation Sequencing (NGS) methods has opened a new area in the use of metabarcoding (Pompanon *et al.* 2011). Actually, the NGS makes it possible to obtain a large quantity of data per sequencing run and by comparing each NGS sequence to the barcodes of a reference barcoding library, allowing the identification of the taxonomic composition of the natural community (Rimet el al. 2016). However, it was recently proved that, on natural diatom communities (e.g. Kermarrec *et al.* 2013a), some barcodes yield better results than others.

Metabarcoding combined with high-throughput sequencing (HTS) has great potential for next-generation biomonitoring applications but requires standardization. For instance, Vasselon *et al.* (2017) have provided a benchmark for the first step of this full process, finding a DNA extraction method, among 5, that provide high DNA quantity at a lowest cost: the SA-Gen method. However, they also observed that different DNA extraction methods gave variations on the relative abundances of some taxa within *Nitzschia, Amphora, Encyonema, Gomphonema*, and *Navicula*, probably due to the efficiency of the lysis methods to disrupt diatom cells.

Although taxonomic assignation was not always stringent, barcoding approaches offer promising perspectives for high throughput screening of diatom diversity, and may represent a powerful tool for biomonitoring in the future (Morin *et al.* 2016). Taking this into account,

progress in diatom classification will come from the combination of molecular techniques with microscopic observations, especially in the case of complicated species complexes (Kermarrec *et al.* 2013b) such as the case of cryptic species (e.g. Evans *et al.* 2008; Mann *et al.* 2008).

4.2 The incongruity of the established arsenic thresholds

Risk assessment is based on the fact that a toxic compound will cause environmental concern when the range of potential toxicity (based on laboratory studies) and the range of real exposure overlap. In European polluted rivers, arsenic concentration ranges approximately between 4.50 and 45 µg As L^{-1}, and it may reach up to 7900 µg As L^{-1} under the influence of mining activities, with an average value of 138 µg As L^{-1} (Smedley and Kinniburgh 2002). As was already explained on ***Chapter 1***, the Aquatic Life Criteria (US EPA 2014) establishes two limits of arsenic concentration in freshwaters: one, set at 340 µg L^{-1} for the Criteria Maximum Concentration (CMC), refers to acute arsenic exposure; while the Criteria Continuous Concentration (CCC), that refers to chronic arsenic exposure, is set at 150 µg L^{-1}. However, toxicity results do not support neither the CMC (see, for instance, the results of ***Chapter 1***) nor the CCC (e.g. Tuulaikhuu *et al.* 2015), indicating that these thresholds should be updated. In the same way, arsenic exposure thresholds for environmental health are much higher than those established for human health, corresponding to 10 µg L^{-1} by the Drinking Water Directive (Council Directive 98/83/EC), being previously set at a *feasibility* threshold of 3 µg L^{-1}, but finally rectified after considered being very expensive to target it (Sauvé 2014). Therefore, the difference in thresholds between environmental and human health should be also considered and updated, recognizing the strong consequences of the actual thresholds on the ecosystem functioning and, indirectly, on the human health.

4.3. Future research needs on arsenic biogeochemistry in freshwater systems

The two different examples of arsenic-impacted sites exposed in the sub-section 3 of the ***General Introduction***, the Pampean streams and the Anllóns River, can be used to exemplify the future research needs on arsenic biogeochemistry in freshwaters.

For instance, high arsenic concentrations are found in the surface waters of the Pampean streams, and this is naturally occurring. This is considered an important health issue, but little is known about its environmental impact. Arsenic bioaccumulation in periphyton is comparatively high among other aquatic organisms (López *et al.* 2016). However, the role that microorganisms play on the As-toxicity to higher trophic levels has been poorly addressed and further investigations should elucidate these questions. Moreover, since aquatic organisms are chronically exposed to high arsenic concentration in aquatics systems as the Pampean streams, microbial communities should be adapted and, in that case, this adaptation may also have a cost. Molecular biotechnology may contribute to understand arsenic resistance mechanism. For instance, analyzing the *"ars"* genes related to arsenate reductase. Research should also focus on the role of other genes like phytochelatin synthase (pcs) gene of microalgae in arsenic toxicity management (Pandey *et al.* 2012; Wang *et al.* 2015). While the

study of genes of resistance in microbiology is rather common and more related to heterotrophic (especially bacteria) than phototrophic organisms, it has been poorly applied in microbial ecotoxicology (Guasch *et al*. 2017).

Regarding arsenic speciation, it is expected that it may experience great temporal changes. However, no studies on arsenic daily speciation changes have been performed. Taking into consideration that epilithic biofilms can reduce and methylate arsenic, it is very important to understand if changes in daily metabolism of algae may influence arsenic speciation in the water column, therefore affecting As-toxicity to other organisms. Biofilm metabolism can change water pH in its highest point of productivity, and it is well documented that arsenic sorption to Fe oxides is weak at high pH (Dzombak and Morel 1990).

The methods used for the determination of the arsenic-species in surface waters have been widely discussed. The fact that arsenite is quickly oxidized to arsenate in oxygenated conditions could derive in As^{III} underestimation. Watts *et al.* (2010) performed *in situ* separation of arsenic species in superficial waters and found an important contribution of the trivalent species, suggesting that new approach for sampling and determination of arsenic species is needed as, for instance, the use of solid-phase extraction (SPE) cartridges in the field to prevent changes in arsenic speciation that can occur between collection and analysis. However, and although some authors consider the use of DGT devices a good option to overcome these limitations, the possibility of determining species of arsenic in DGT extracts is in some cases unclear (Bennet *et al.* 2011). In addition, many determination techniques of arsenic-species does not account for the presence of organoarsenicals. Therefore, it is possible that organic arsenic species, if present, may contribute to the total “inorganic arsenic” measurement by some *in situ* techniques such as some kind of DGT (Bennet *et al.* 2011). Future research should focus on the application of new approaches for the investigation of arsenic speciation, including organoarsenicals, especially in highly productive areas.

In contrast to the Pampean streams, arsenic concentration is very low in the Anllóns River water. In this case, the presence of arsenic-polluted sediments caused by old gold-mining activities puts into question its mobility and the role that biofilms play on it. Several investigations referenced in this thesis demonstrated that both epilithic and epipsammic biofilms play a key role on arsenic retention and biotransformation, and that phosphate modulates the toxicity and mobility of arsenic. It has been shown that freshwater biofilms may methylate and detoxify arsenic but it is not clear to which extent and the role that microalgae and prokaryotes play on this detoxification (Bertin *et al.* 2011), even which are the set of conditions that may trigger arsenic mobilization, biotransformation and the resulting toxicity. In the last few years, a huge amount of genomic sequences has been published in databases, including a complete characterization of several bacteria metabolizing arsenic (Muller *et al.* 2007; Arsène-Ploetze *et al.* 2010). In this respect, metagenomic approaches based on high-throughput technologies may be of great interest since they allow investigating the structure and function of the whole community (Bertin *et al.* 2011). Moreover, advanced analytical procedures capable of

quantifying arsenic speciation in water, sediment and biota are also needed. On top of that, little is known about the impact of human activities such as the discharge of high organic matter and/or nutrients in arsenic speciation and mobility in freshwater systems like the Anllóns River. The role of dissolved organic matter, mostly constituted by humic and fulvic acids, has not been well studied. It is known that humic substances can bind to arsenic making it unavailable for organisms (Sharma and Kappler 2011). Arsenic binding to humic acids depends on pH and type of humic acid (Buschmann *et al.* 2006), but its effect to the biofilm community has not been studied. Finally, and as well commented all along this thesis, nutrient availability has a strong influence on arsenic toxicity to freshwater algae and biofilms, which may retain arsenic and transform it into more or less toxic forms depending on phosphate conditions, and influence on the toxicity to other higher organisms like fish. However, it is still uncertain how PO_4^{3-} influences the uptake, retention and transformation of arsenic species in microalgae or biofilms. More detailed studies are needed to solve these uncertainties. Considering the stoichiometry of P in relation to other elements like N, which is intimately linked with P, could be crucial to understand the dynamics of P and As uptake and toxicity in microorganisms (MacNeill, personal communication, May 5, 2017), and future investigations should also move along on this line. It is also crucial to perform new experiments to be able to conclude under which conditions biofilms protect or not fish from arsenic toxicity. To understand this challenging issue, it will be necessary to explore a larger range phosphate and arsenic concentrations, considering also the arsenic speciation, in higher complex experiments. In that way, the studies of this thesis help to set out future questions and experiments of higher complexity.

The intention of this thesis is to contribute to the understanding of the arsenic biogeochemistry in freshwaters, highlighting the key role that biofilms play on it, also their effects due to the toxicity and their influence on the toxicity to other aquatic organisms such as fish, probably causing changes on the ecological status of the fluvial systems. We consider that all mentioned future perspectives and research needs may have significant contributions to move along on these lines.

5. General Conclusions

1. Short-term biofilm exposure to environmentally realistic arsenic concentrations (130 µg As L^{-1}) and under P_limited conditions may cause important toxic effects to biofilms, becoming **less phototrophic** after being reduced the algal growth and productivity. Moreover, arsenic may inhibit the algal succession process in biofilms, causing changes in the algal community. A **loss of diatom species (those sensitive to arsenic)** and a **significant decrease in their cell size** may allow diatoms to become **more tolerant** to the toxicant than the other algal groups.

2. Similar effects may be observed in epilithic biofilms growing in a **mining impacted river**, even being the toxicant mostly associated to sediments. These biofilms **accumulate** high arsenic concentrations, resulting in a **inability of algae to grow** and in an **increase in bacterial and dead diatom density**. Therefore, **the release of arsenic** (through phosphate replacement or microbial activity) from sediments to other compartments such as water and/or biofilms should be contemplated in such mining areas, especially in rural regions where **phosphate inputs** are important. Other environmental factors in field experiments (such as nutrients, DOC, temperature or light availability) must be also taken in consideration when analyzing the arsenic effects in freshwater ecosystems.

3. **Methylated As-species (especially, DMA^V)** may be found within arsenic affected biofilms, suggesting arsenic detoxification (methylation) by microorganisms, even under eutrophic conditions, what agree with other field studies but not with some laboratory studies and suggested theoretical models, contributing thus to the lack of consensus about the role of nutrients (mainly P) on arsenic uptake and speciation by microorganisms.

4. Further experiments are needed to disentangle and **better understand the complex set of processes contributing to arsenic and phosphate cycling by microorganisms.** Considering the stoichiometry of P in relation to other elements like N could allow a better understanding of the dynamics among P and arsenic uptake and toxicity in microorganisms.

5. We strongly support the use of biofilm and a **multi-endpoint approach** to analyse effects of toxicants in freshwater ecosystems, especially including the measure of the **chlorophyll-a fluorescence** in biofilms and the **diatom biovolume (cell size)**. Regarding fish endpoints, **changes in complex behaviors** are practical, ecologically relevant measures of toxicological effects, and **aggression** in particular should be considered in assessment of arsenic impacts as it is a highly dynamic and responsive process that may show immediate impacts and can influence several other aspects of behavior. Also, the analysis of arsenic speciation in the **extracellular** and **intracellular** part of the biofilm is highly recommended and contribute to the understanding of the arsenic cycle in freshwaters.

6. **Multi-trophic studies** are crucial to better elucidate the **real** effects of toxicants. Such **multidisciplinary**, cross-taxon research should therefore be considered for understanding the impacts of arsenic toxicity on aquatic ecosystems. An important finding in this respect from this thesis is the **aggravating influence of fluvial biofilms on the impacts of arsenic exposure in fish**.

7. Exploring **new endpoints** along with the traditional taxonomical parameters can greatly enhance the evaluation of fluvial ecosystem quality for biomonitoring practices using diatoms. In this sense, the **easiness**, **quickness**, **cheapness**, **global acceptation** and **no especial training in diatom taxonomy** should be the main characteristics of these new endpoints. Moreover, progress in diatom classification will come from the combination of **molecular techniques** with microscopic observations, especially in the case of complicated species complexes such as the case of cryptic species.

8. The results obtained in this thesis about the arsenic effects in fluvial systems **call into question the limits** of arsenic concentration established by the US EPA (2014) for freshwater systems. Also, the **difference in thresholds** between environmental and human health should be considered and **updated**, recognizing the strong consequences of the actual thresholds on the ecosystem functioning and, indirectly, on human health.

9. This thesis provides valuable information to understand **the contribution of benthic biofilms to arsenic biogeochemistry** in fluvial environments, and specifically in the water-biofilm interface.

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concentration is reduced by algae, this may be counterproductive at an ecosystem scale.

For mosquitofish, the effects of arsenic exposure are overall detrimental. Despite the increased biomass seen here with arsenic, bioaccumulation of arsenic is harmful (de Castro et al., 2009; Moeller et al., 2003; Sopinka et al., 2010) and increased aggression may increase the chance of physical damage (e.g. Huntingford and Turner, 1987) and exacerbate physiological effects of arsenic exposure (e.g. Scott and Sloman, 2004). Moreover, in addition to, or as a consequence of, the effects documented here other functions and interactions are likely to be disrupted. For example, both mate recognition (e.g. Fisher et al., 2006) and predator recognition (e.g. Mandrillon and Saglio, 2007) are compromised by alteration of the chemical environment. The mechanisms underlying the behavioural changes demonstrated in this study may involve sensory, hormonal, neurological and metabolic systems (Scott and Sloman, 2004) all of which may also affect other behaviours including locomotory behaviours like predator avoidance or swimming performance. The increase in aggression and lack of effects on feeding behaviour in this study suggest locomotory functions were not affected. However, the exposure treatments here were neither particularly acute nor chronic and increased exposure concentrations or durations are likely to lead to more serious impacts. Finally, here we used an invasive, highly tolerant fish as a model. The effects of arsenic exposure on potentially endangered native species would be both more difficult and more critical to evaluate.

In conclusion, we have shown here that changes in complex behaviours are practical, ecologically relevant measures of toxicological effects (e.g. Scott and Sloman, 2004; Weis et al., 2001). Aggression in particular should be considered in assessment of arsenic impacts as it is a highly dynamic and responsive process that may show immediate impacts and can influence several other aspects of behaviour. In common with other authors, we also highlight interacting effects of contaminant exposure, both through integration of behavioural and physical mechanisms (e.g. Scott and Sloman, 2004; Weis et al., 2001) and consideration of different taxa together (e.g. Scott and Sloman, 2004; Weis et al., 2011). In particular, toxicant responses in multi-trophic, natural ecosystems are often found to be different from single-species laboratory studies. Multi-trophic studies are therefore crucial to elucidate the real effects of toxicants. An important finding in this respect from the current study is the aggravating influence of algae on the impacts of arsenic exposure in fish. Bioremediation of arsenic contaminated waters using aquatic algae should therefore be carried out with consideration of entire ecosystem effects. Such multidisciplinary, cross-taxon research is crucial for understanding the impacts of arsenic toxicity and thus restoration of aquatic ecosystems.

Conflict of interest

The authors declare no conflict of interest.

Contributors

Concept: HG, KM, EGB; experimental design: HG, KM, EGB; field collection: EGB, KM, HG; carried out experiments: KM, LBF, MR, GU, HG; video analyses: KM, PS; biochemical analyses: LBF, MR, PS, GU, HG; statistical analyses: KM; wrote the paper: KM; edited, revised, critiqued and wrote small sections of the manuscript: HG, EGB, MR, LBF, GU.

All authors have approved the final article.

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